REMARKS

Reconsideration is requested.

Claims 1, 4, 9, 10, 13-16, 33, 36, 41 and 45-50 are pending.

The Section 103 rejection of claims 1, 4, 9, 10, 13-16, 33, 36, 41 and 45-50 over Field (U.S. Patent No. 6,593,140) and Gorfien (U.S. Patent Application Publication No. 2006/0148074) is traversed. Reconsideration and withdrawal of the rejection are requested in view of the attached evidence and the following remarks.

The Examiner asserts that the "media requirements [of Field] overlap that of the instant claims". See page 2 of the Office Action dated April 5, 2011. The Examiner concludes that one would expect the methods of Field to be as successful as that of the instant claims. Id.

There is no overlap between the media requirements of this art and the instant claims. Where Field culture myeloma cells in 0.2 mg/l of ferric ammonium citrate (FAC), the present application requires a minimum of 0.4 mg/l FAC (such as in claim 10) or the equivalent iron concentration (i.e. 0.064 mg/l iron, such as in claim 1).

The applicants understand the Examiner to believe that Gorfien discloses the use of amounts of iron falling within the range of present claim 1 to culture myeloma cells and that it would have allegedly been obvious to have used the FAC taught by Field in the method of Gorfien.

The applicants submit however that Field teaches that hybridoma cells when cultured under agitated conditions are destroyed as the FAC concentration is increased

over 0.1 mg/L, when culturing takes place in the absence of transferrin or a chelator. The applicants submit that this is illustrated in Fig 2B of Field reproduced by the Examiner on page 6 of the Office Action dated April 6, 2011, where the number of cells falls from $2x10^{-5}$ when the FAC concentration is 0.1 mg/L to 0 when the FAC concentration is 10 mg/L. A reduction in the number of cells in the culture cannot be equated with growth of the cells, as is required by the instant claims. "One would expect the same results to occur for myeloma cells." See page 6 of the Office Action dated April 6, 2011

Field suggests that this is exactly the case, in that Example 5 illustrates that myeloma cells fail to thrive when cultured in the absence of transferrin or a chelator at 0.2 mg/L FAC.

Failure to thrive is the direct opposite of the claimed requirement for growth of the myeloma cells. The meaning of "thrive" is to grow or increase in bulk or stature or to grow vigorously or luxuriantly. Failure to thrive means that this growth does not happen.

The Field Declaration addresses the Examiner's point. The person skilled in the art at the time of this application considered hybridoma and myeloma cells to have the same metabolic requirements. Hence, as stated by the Examiner, the person skilled in the art would expect myeloma cells to react in the same way as demonstrated by Field for hybridoma cells.

The skilled person would not therefore consider increasing the concentration of iron in the medium, as is proposed by Gorfien because Field demonstrates that

increasing the FAC concentration results in cell death. The person skilled in the art would know, from the disclosure of Field that FAC would not be suitable at concentrations above 0.1 mg/L for the culture of hybridoma and hence myeloma cells under agitated conditions. As is also described in the Field Declaration, the conditions under which the cells are cultured are also critical.

Furthermore, as is also described in the Field Declaration, Gorfien found it essential to mitigate the known toxic effects of high iron concentrations by using beta-glycerophosphate.

Given the disclosure of Field and Gorfien it is all the more surprising that the present inventors were able to find a particular range of iron and FAC concentrations under which myeloma cells can be successfully cultured and in which they can thrive.

Withdrawal of the Section 103 rejection is requested.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. The Examiner is requested to contact the undersigned, preferably by telephone, in the event anything further is required.

Respectfully submitted,

NIXON & VANDERHYE P.C.

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THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Matthew David Osborne and Jonathan H. Dempsey,

: Examiner. Maria Marvich

Application No. 10/567,453

: Group Art Unit: 1633

Filing Date: July 18, 2006

: Atty Docket No.: BJS-620-412

For: MYELOMA CELL CULTURE IN TRANSFERRIN-FREE LOW IRON MEDIUM

DECLARATION OF NON-OBVIOUSNESS

UNDER 37 C.F.R. s103

I, Raymond Field, hereby declare that:

1. I am a citizen of the UK residing in Melbourn, Cambridgeshire, UK.

2. I received a degree B.Sc.(hons) Biochemistry, University of Warwick in 1981 and PhD. University of Glasgow, Dept. Biochemistry in 1985. I have subsequently been employed in industrial biotechnology and biopharmaceutical companies until the current time. I was a group leader in Cell Culture Development in Celltech Biologics (UK) 1985-1993, in Protein Expression Group at AstraZeneca 1993-1994, then Head of Cell Culture Development/Cell Sciences at Cambridge Antibody Technology / MedImmune until current date.

3. The details of my educational and professional history are set forth in my Curriculum Vitae, which is attached hereto as Exhibit A.

4. I am a member of the following professional organizations. European Society of Animal Cell Technology. (ESACT).

5. In addition, I have 26 years of experience in Production of biopharmaceuticals from mammalian cell culture and I am the author or co-author of more than 12 scientific journal articles

on the subject of recombinant protein expression and cell culture in serum-free media. A list of my publications is included in Exhibit A. My current area of research involves production of recombinant proteins from CHO and myeloma cell lines.

6. I am named as an inventor or co-inventor in issued patents/patent applications.

US5681718. Methods for Enhanced Production of Tissue Plasminogen Activator in Cell Culture using Alkanoic Salts Thereof. Inventor: Raymond Paul Field.

US6413746. Production of Proteins by Cell Culture. Inventor: Raymond Paul Field.

US6593140 & WO94/02592. Animal Cell Culture. Inventor: Raymond Paul Field.

US6660501. Production of Proteins by Cell Culture. Inventor: Raymond Paul Field.

7. I am appointed as an industrial steering group member of the Bioprocessing Research and Industry Club of the Biotechnology and Biological Sciences Research Council, co-responsible for reviewing grant proposals in the area of Bioprocessing.

8. I am familiar with the content of US patent application number 10/567,453 since the inventors were members of my group at the time when the laboratory experiments were being performed and the patent was filed. I have reviewed the Final Action dated April 6, 2011 on US patent application number 10/567,453.

9. Based on my review of the April 6, 2011 Final Action, I understand the Examiner to have rejected claims 1, 4, 9, 10, 13-16, 33, 36, 41 and 45-50 as allegedly being obvious over Field. (US 6,593,140) in view of Gorfien *et al.* (US 20060148074). Specifically, the Examiner asserts that it would be obvious to use ferric ammonium citrate as taught by Field in the media taught by Gorfien *et al.* because Gorfien *et al.* teach that it is within the ordinary skill of the art to use particular levels of iron to culture myeloma cells and because Gorfien *et al.* teach that it is within the ordinary skill of the art to use ferric ammonium citrate as a source of iron.

10. As a scientist having considerable knowledge, skill and experience in the field of the invention of the '453 application, and as an inventor of the invention claimed in Field., I do not believe that the combined disclosures of Field and Gorfien *et al.* would motivate one of ordinary skill in the art to culture a myeloma cell line in a medium in which iron is present at concentrations

of 0.064 mg/L to 1.6 mg/L, wherein the medium does not contain transferrin, a lipophilic chelator, a synthetic nitrogen-containing chelator or a lipophilic synthetic nitrogen-containing chelator, wherein the source of iron is a soluble iron compound selected from the group consisting of ferric ammonium citrate, ferric ammonium oxalate, ferric ammonium fumarate, ferric ammonium malate and ferric ammonium succinate.

11. The bases for my above-stated conclusion are as follows:

Field shows in Figure 2A that high concentrations of iron in the culture medium are required in order to transport iron into hybridoma cells in culture in <u>static</u> flasks in the absence of either transferrin or lipophilic iron chelator (e.g. tropolone). However in cultures of hybridoma cells that are <u>shaken or agitated</u> (to simulate a fermenter/bioreactor environment) it was shown by Field that <u>for the same hybridomas,</u> high Fe concentrations in the absence of transferrin or a chelator resulted in cell death (Fig 2B). This was the basis for Field's use of tropolone to supply iron to hybridoma and other cells in culture by only using a low iron concentration.

12. It was a surprising and unexpected discovery that although hybridoma cells (a fusion of a myeloma cell and a B lymphocyte) are destroyed by higher levels of Fe supplied as Ferric Ammonium Citrate (FAC) in agitated suspension culture in the absence of transferrin or a chelator (as taught by Field), myeloma cells (one fusion partner of a hybridoma) can thrive and grow with the same Fe levels under similar conditions, as is shown in the instant application. This was surprising since hybridoma cells and myeloma cells in all other aspects of culture process parameters behave essentially identically to each other, and often differently to many other cell types (e.g. CHO cells). An example of the difference in requirements between hybridoma and myeloma cells on the one hand and CHO cells on the other is the absolute requirement for supply of glutamine to both myeloma and hybridoma cell lines to maintain cell viability, whereas CHO cells can tolerate the absence of glutamine.

13. The similarity in cell metabolism between hybridoma and myeloma cells lies behind the success of the myeloma (NS0) expression system for production of commercial quantities of recombinant proteins and this was due to the ability to apply the industrial serum-free cell culture processes and media formulations previously used for mouse hybridoma cells directly to the myeloma (e.g. NS0) cell processes. This similarity also extends to the metabolism of amino acids vitamins and key trace elements by both hybridoma and myeloma cells (for example see. Biblia

and Robinson 1995, Biotechnol. Prog. 11:1-13 and references cited therein.). If anything, myeloma cells were considered more fragile and fastidious than hybridoma cells for industrial scale culture, for example due to their requirement for addition of exogenous sterols. Given these significant similarities in cell metabolism and nutrient requirement, those of us working in this field thought that NS0 cells would be equally sensitive to how the Fe was supplied in agitated suspension cultures as were hybridoma cells.

14. It was surprising that this turned out not to be true as demonstrated in experiments using mouse NS0 cells which was the basis for the patent application of Osborne and Dempsey.

15. With regard to Gorfien *et al.*, this publication does not make such distinctions between cell types and includes a long list of potential cell types for which their media could be used, although in fact there is only one demonstrated example of use with HEK-293 cells and the remainder are with CHO cells. There is no example with myeloma or hybridoma cells, although the supply of lipid mixtures to NS/0 cells is mentioned by Gorfien *et al* [Paragraph 0144]. However, no distinction between static or agitated suspension cultures is mentioned. This is crucial. Anyone skilled in the art would attest to the preference for culturing the more robust CHO cells in an agitated industrial bioreactor system due to their shear resistance and the ease with which high viability cultures can be maintained even in a lower nutrient environment. The greatest challenges with CHO cells were around their ability to grow homogeneously in suspension culture, which of course is not an issue with the myeloma and hybridoma cells that are 'natural' suspension cells. One should also distinguish between cells that will propagate easily in suspension culture e.g. in a static or low agitation environment, and the more highly mixed and stirred environment that represents an industrial bioreactor or a simulation thereof in flask cultures. This important distinction in the environment is not made by Gorfien *et al* and hence it does not address the core issue that Osborne and Dempsey do in their patent specification.

16. Furthermore Gorfien *et al* include compounds such as beta-glycerophosphate in their media formulations that are widely known to detoxify the effects of high iron concentration. For example the detoxifying effects of beta glycerophosphate are reported by Rasmusen and Toftlund (In Vitro Cell Dev Biol. 1986 Apr; 22(4):177-9, copy attached). Beta-glycerophosphate is present at high concentrations (0.9g/L) in the media formulations of Gorfien *et al.*, as described in the Salts II section in Table 2 on page 16 of Gorfien *et al.* So the Gorfien *et al.* teaching is that a

detoxifier must be included in the medium to mitigate any toxic effects of a high iron concentration in a suspension culture environment.

17. Example 5 of Field teaches that in the absence of tropolone or transferrin, but in the presence of 0.2 mg/l ferric ammonium citrate, myeloma cells failed to thrive and died within 48 hours. Failure to thrive suggests that, whilst there may be some viability of the myeloma cells, there is no growth in this example of Field. If, knowing from Field that myeloma cells fail to thrive in low iron concentrations, Osborne and Dempsey had decided simply to increase the iron concentration as the Examiner proposes Gorfien *et al* would suggest they do, (despite knowing from Field that high iron concentrations of iron are toxic to hybridoma cells and would be expected to be similarly toxic to myeloma cells known to have the same metabolic requirements) they would also take from the Gorfien *et al* publication that those toxic effects of a high iron concentration must be mitigated using beta-glycerophosphate. However I note that Osborne and Dempsey have shown that they do not have to resort to using detoxifying compounds such as beta-glycerophosphate, and instead just added simple phosphates and added the iron in the form of Ferric Ammonium Citrate.

18. In my view it was not obvious to try the approach of Osborne and Dempsey even with the knowledge of Gorfien *et al.* and Field. for myeloma cells cultured in a high agitated suspension culture environment.

19. Therefore, I do not believe that a method to culture a myeloma cell line in a medium in which iron is present at concentrations of 0.064 mg/L to 1.6 mg/L, wherein the medium does not contain transferrin, a lipophilic chelator, a synthetic nitrogen-containing chelator or a lipophilic synthetic nitrogen-containing chelator, wherein the source of iron is a soluble iron compound selected from the group consisting of ferric ammonium citrate, ferric ammonium oxalate, ferric ammonium fumarate, ferric ammonium malate and ferric ammonium succinate is obvious over Field in view of Gorfien *et al.*

20. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such

wilful false statements may jeopardize the validity of the '158 application or any patent issued thereon.

5th July 2011 R. P. Field

Date

Attachment A
CURRICULUM VITAE

Name: **Raymond Paul Field**

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2007- Present
MedImmune Ltd, Cambridge UK

Director of Cell Sciences and CMC Team Leader
MedImmune Ltd*
Milstein Building,
Granta Park,
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CB21 6GH

Experience:

Process Validation for BLA submission of a mAB project.

Drug and Process development for recombinant protein products for CTA/IND submission (6+ programmes to CTA submission, transferred to CMO and US Biopharma)

Preparation of expert reports and briefings for legal and patent attorneys (e.g. litigation and manufacturing IP opposition hearings).

Commercial negotiation and implementation of new technology external collaborations within budget

Authoring and review of documents and reports CMC CTA/IND and BLA submissions.

Key role in due diligence activities for in and outlicensing.

Presentation of CAT's science and Development capabilities at international conferences and forums.

Joint responsibility for feasibility studies of GMP facility design study and CMO evaluations.

Streamlining of interface and project & technology transfers between discovery and development groups.

November 1994-2007
Cambridge Antibody Technology, Cambridge UK.
Senior Scientist Development
Head of Mammalian Cell Technology
Director Cell Sciences

Jan 1993-Oct 1994
Zeneca Pharmaceuticals (now AstraZeneca), Alderley Park, Macclesfield.

Head of Lab: Recombinant Protein Production, Research Division.

Responsibilities:
- Expression system and process development of mammalian and insect cell systems for supply of recombinant proteins for drug discovery screens
- Leading direction of technology for protein production and team of 3 scientists

Feb 1985-Jan 1993
Cell Culture Development Division, Celltech Biologics Ltd, Slough, UK.

Group Leader, Cell Culture Development

Responsibilities: Process Development and Scale up of mammalian expression systems for Recombinant Protein Production. Use and development of CHO and myeloma expression systems, rational serum-free process development and cell line development for scale up to GMP production (ultimately to 2000L scale).

EDUCATION:

Oct 1981-Feb 1985 PhD, University of Glasgow, Dept. Biochemistry
Primary Culture of Uterine Cells: Markers of Growth and Differentiation.

1978-1981 B.Sc.(hons) Biochemistry, University of Warwick

PATENTS:

Methods for Enhanced Production of Tissue Plasminogen Activator in Cell Culture using Alkanoic Salts Thereof. US5681718. Inventor: Raymond Paul Field. Date of Patent Oct28 1997

Production of Proteins by Cell Culture.
US 6413746 Inventor: Raymond Paul Field. Date of Patent Jul 2 2002.

Animal Cell Culture.
US 6593140 & WO 94/02592 Inventor: Raymond Paul Field. Date of Patent: Jul 15 2003.

Production of Proteins by Cell Culture:
US 6660501 Inventor Raymond Paul Field. Date of Patent: Dec 9 2003

PUBLICATIONS:

Numerous presentations and Invited presentations at international conferences.
IBC (various), ESACT, IIR, Cell Culture Engineering etc.

Field R (2007), Recombinant human IgG production from myeloma and chinese hamster ovary cells. Chapter (pp57-80) in "Cell culture and Upstream Processing", Ed. M Butler, pub. Taylor and Francis Group.

Daramola, O., Hatton, D. and Field, R. (2005) Meeting the challenges of IgG expression: from antibody libraries to clinical supply. *Bioprocessing J.* 4: 33 - 37

Dempsey J, Ruddock S, Osborne M, Ridley A, Sturt S, Field R
Improved fermentation processes for NS0 cell lines expressing human antibodies and glutamine synthetase. Biotechnol Prog. 2003 Jan-Feb;19(1):175-8.

Osborne M, Bracewell D, Dempsey J, Field R, Fish B, Ritchie C.
Rapid estimation of Human Monoclonal (IgG4) Concentration in Cell Culture Supernatants. in Animal cell technology From Target to Market, Kluwer Academic Publishers, 2001

Thompson JE, Vaughan TJ, Williams AJ, Wilton J, Johnson KS, Bacon L, Green JA, Field R, Ruddock S, Martins M, Pope AR, Tempest PR, Jackson RH.
A fully human antibody neutralising biologically active human TGFbeta2 for use in therapy
J Immunol Methods. 1999 Jul 30;227(1-2):17-29.

Birch JR, Boraston RC, Metcalfe H, Brown ME, Bebbington CR, Field RP.
Selecting and designing cell lines for improved physiological characteristics.
Cytotechnology. 1994;15(1-3):11-6.

Metcalfe M, Field R.P, Froud S.J.
The use of 2-Hydroxy-2,4,6-Cycloheptarine-1-one (Tropolone) as a Replacement for Transferrin, in: Animal Cell Technology Products of Today, Prospects for Tomorrow, (Proc. 12th ESACT Meeting), Eds. R.E. Spier, J.B. Griffiths and W. Berthold, Butterworth Heinemann, Oxford, 1994

Brown ME, Renner G, Field RP, Hassell T.
Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system

Cytotechnology. 1992;9(1-3):231-6.

Hassell T, Brand H, Renner G, Westlake A and Field R P.
Stability of Production of Recombinant Antibodies from Glutamine Synthetase Amplified CHO and NSO Cell Lines. in Animal Cell Technology: Developments, Processes and Products eds Spiers R.E. et al. Butterworth Heineman 1992.

Field R P, Brand H, Renner G L, Robertson H A and Boraston R
Production of a Chimeric Antibody for Tumour Imaging and Therapy from Chinese Hamster Ovary (CHO) and Myeloma Cells. Proceedings of The 10th ESACT Meeting May 1990.pp 742-744

Field, R Abstracts of Papers - American Chemical Society (1988) 196:141
Suspension Growth of Recombinant CHO Cells in Serum-Free and Glutamine-Free medium

Field, R, Cockett, M & Froud S J in :"Advances in Animal Cell Biology and Technology for Bioprocesses" eds Spier, R E et al (1988) "Glutamine Synthetase Amplification of TIMP Expression in CHO cells"

Field, R.P. & Leake R.E. Biochem. Soc. Trans. 12:258 (1984)

Field, R P & Leake R.E. Biochem. Soc. Trans. 12:319 (1984)

PubMed

Display Settings: Abstract

In Vitro Cell Dev Biol. 1986 Apr;22(4):177-9.

Phosphate compounds as iron chelators in animal cell cultures.

Rasmussen L, Toftlund H

Abstract
We have studied the capacity of a number of phosphate compounds to act in the double role as a phosphate source and a detoxifier of ferric chloride hydroxo compounds, i.e. as Fe(III) chelators. The tested compounds were: orthophosphate, trimetaphosphate, alpha-glycerophosphate, beta-glycerophosphate, phytic acid, and phosphorylcholine; the test organism the ciliate protozoon Tetrahymena thermophila, an animal cell, and the nutrient medium was synthetic, consisting solely of low-molecular-weight compounds. We assessed growth rates of cells in two experimental series. First, phosphate-starved cells were exposed to the tested phosphate compound as the only phosphate source and the ferric chloride concentrations were varied stepwise from 0 to 1000 microM. Second, we offered the cells orthophosphate as a phosphate source and selected phosphate compounds as chelators. The cell growth results allow the following conclusions: orthophosphate, trimetaphosphate, alpha-glycerophosphate, and beta-glycerophosphate are excellent phosphate sources; trimetaphosphate, alpha-glycerophosphate, beta-glycerophosphate, and phytic acid are excellent Fe(III) chelators; of the tested compounds trimetaphosphate, alpha-glycerophosphate, and beta-glycerophosphate are excellent in the double role as a phosphate source and a ferric chloride hydroxo detoxifier, i.e. as a Fe(III) chelator.

PMID: 3700321 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

LinkOut - more resources

Biotechnol. Prog. **1995**, *11*, 1–13

TOPICAL PAPER

In Pursuit of the Optimal Fed-Batch Process for Monoclonal Antibody Production

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Fed-batch culture currently represents the most attractive choice for large scale production of monoclonal antibodies (MAbs), due to its operational simplicity, reliability, and flexibility for implementation in multipurpose facilities. Development of highly productive cell lines, maximization of cell culture longevity, and maintenance of high specific antibody secretion rates through genetic engineering techniques, nutrient supplementation, waste product minimization, and control of environmental conditions are important for the design of high-yield fed-batch processes. Initially simple supplementation protocols have evolved into sophisticated serum-free multinutrient feeds that result in MAb titers on the order of 1–2 g/L. Limited research has been published to date on the effects of various culture parameters on potentially important quality issues, such as MAb glycosylation and stability. Although most fed-batch protocols to date have relied on relatively simple control schemes, increasingly sophisiticated algorithms must be applied in order to take full advantage of the potentially additive effects of manipulating nutrient and environmental parameters to maximize fed-batch process productivity.

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Introduction

Monoclonal antibodies (MAbs) are finding increased markets for use as diagnostic reagents, *in vivo* imaging agents, and therapeutics. A recent report indicates that total MAb product revenues in the United States approached $600 million in 1992 and could possibly rise to nearly $4 billion by 1998 (*Genetic Engineering News*, 1993). Many of the experimental MAb therapeutic strategies call for high *in vivo* dosages, ranging from 0.5 to more than 5 mg/kg (Aulitzky et al., 1991). Provided that these MAbs capture a reasonable size market, for example 1 million doses per year, production scales of tens to hundreds of kilograms per year would be required. It is this prospect that provides the motivation to develop high-level expression systems for monoclonal antibodies, in order to reduce both the capital for a proposed production facility as well as operational costs.

Nutrient fortification of batch or supplementation in fed-batch culture has been widely used to improve antibody yields. Since final MAb titers are determined by cell culture longevity and specific MAb secretion rate, fed-batch process development strategies aim at maximizing these two parameters in order to maximize final MAb yields. A good measure of cell culture longevity is the final integrated number of viable cells over the course of the culture, also known as the culture viability index (Luan et al., 1987a). For many cultures, particularly those where the specific MAb secretion rate remains constant, increases in the culture viability index directly translate into an increase in the final antibody yield.

As will be discussed in this review, initial attempts to maximize culture longevity by feeding cultures with only a few nutrients, such as glucose and glutamine, today have evolved into multifeed strategies that result in final antibody titers of 1–2 g/L. Many of these high-yielding processes have begun to exploit the combined effects of manipulating both nutrient feed composition and environmental conditions to increase culture longevity and specific secretion rates. The impact of such manipulations on product quality is just now being explored. Algorithms and on-line probes that can be used to control and monitor such processes are being developed. The combined application of these research efforts will result

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Atty. Ref.: 620-412
Response After Final Rejection
July 6, 2011
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Theodora Bibila received her bachelor's degree in chemical engineering from the Aristotle University of Thessaloniki, Greece, and her M.S. and Ph.D. degrees in microbial and chemical engineering, respectively, from the University of Minnesota. Her doctoral thesis advisor was Professor Michael Flickinger. She is currently a Research Fellow in the Bioprocess Research and Development Department at Merck Research Laboratories. Her main responsibilities include small and pilot scale research for the development of optimized large scale processes for the production of proteins and viral vaccines from mammalian cell culture.

David Robinson received his bachelor's degree in chemical engineering from the University of California at Berkeley and his doctorate from the Massachusetts Institute of Technology. He conducted his thesis research with Professor D. I. C. Wang and his postdoctoral research with Professor Klaus Mosbach at the E.T.H Zürich and Dr. Arthur Einsele at Sandoz. He is currently a Research Fellow in the Department of Cellular and Molecular Biology at Merck Research Laboratories. His principal responsibilities include the development of cell lines, media, and processes for the production of therapeutic proteins and viral vaccines in animal cell culture.

in high-productivity fed-batch processes, whose final titers and productivities will rival those achieved in high-density cultivation systems. Most importantly, a simple stirred-tank bioreactor can be used for the type of fed-batch process described in this paper. This type of bioreactor has been well studied and scale-up parameters are well understood.

Cell Line and Medium Development

The most essential component of any cell culture process is the development of a stable cell line that secretes the desired product at high rates. The specific rates of antibody production by hybridoma cell lines are quite variable, ranging from less than 2 up to 80 pg/cell/day (Savinell et al., 1989). Although antibody production is typically non-growth-associated (Miller et al., 1988a; Ray et al., 1989), examples exist of hybridomas that exhibit growth-associated production kinetics (Schurch et al., 1992). In some cases, cellular productivity can be unstable, with specific productivities dropping severalfold within a few months in continuous culture (Frame and Hu, 1990). Higher producing cells can, at times, be selected by single cell cloning of apparently low-producing cells (Murakami, 1990; Seaver, 1992).

In addition to MAb production by hybridoma cells, recombinant gene technology has been used to develop stable cell lines expressing chimeric, humanized, or human antibodies at high levels. Transfection of antibody genes with the selectable and amplifiable marker, glutamine synthetase (GS) (Bebbington et al., 1992), into NS0 myeloma cells generated cell lines secreting human and humanized antibodies at rates of 20–50 pg/cell/day (Robinson et al., 1994a). High-level expression of MAbs has also been achieved in GS-amplified CHO cells, although the production levels of these cell lines in large scale culture are lower than those achieved for GS-amplified NS0 cell lines (Brown et al., 1992). Dihydrofolate reductase (DHFR) selection and amplification in SP2/0 myeloma cells generated a cell line that expressed a chimeric MAb in a growth-associated manner in which the specific productivity increased from 20 to nearly 80 pg/cell/day as growth increased (Robinson and Memmert, 1991). DHFR selection and amplification in CHO cells have led to the establishment of highly productive cell lines that secrete up to 100 pg/cell/day of a humanized MAb (Page, 1991), while sequential transfections and amplification of dual markers, DHFR and adenosine deaminase, yielded CHO cell lines expressing a murine IgM at 30 pg/cell/day (Wood et al., 1990) and a chimeric MAb at 80–110 pg/cell/day (Fouser et al., 1992).

Although hybridomas traditionally have been grown in serum-containing medium, the abundance of serum-free media formulations available today indicates that hybridoma growth in the absence of serum is generally feasible. Serum-free media have been developed by supplementing a standard basal medium, such as RPMI-1640 (Moore and Hood, 1993), Iscove's Modified Dulbecco's medium, a mixture of nutrient mixture F12 and DMEM (Bjare, 1992), and other basal media (Schneider, 1989; Schneider and Lavoix, 1990), and by substituting defined components for serum functions (Maiorella, 1992a). Completely protein-free media have also been developed (Fike et al., 1991; Franek and Dolnikova, 1991). Many of these serum-free formulations support the growth of hybridoma and myeloma cells to more than 1×10^6 cells/mL in standard batch culture, with typical final antibody titers of 10 to more than 100 mg/L. However, the selection of an appropriate basal medium should be done on a cell line basis.

Maximization of cell culture longevity in fed-batch culture systems is achieved by periodic supplementation of the culture with nutrients that are quickly consumed or exhausted. Current analytical techniques allow the analysis of spent cell culture medium to determine nutrient utilization and identify limiting nutrients. Analysis of spent medium for glucose, typically the main carbon source, can be performed using automated analyzers, while amino acid concentrations can be determined by HPLC (Reid et al., 1987). Analysis for other nutrients, such as vitamins, lipids, proteins, and trace elements, is more challenging and time-consuming. Limitations by these nutrients can alternatively be identified by performing add-back experiments and monitoring culture performance (Robinson et al., 1994a). The use of colorimetric, fluorimetric, or other automated cell growth assays, adapted to a microplate format, can substantially reduce the labor associated with this task. Colorimetric

1829956

Table 1. Methods for Increasing MAb Yields

method	cell line	final MAb titer (mg/L)	-fold MAb titer improvement over batch culture	reference
	Nutrient Fortification (Batch Culture)			
	mouse hybridoma 2c3.1	450	8	Jo, 1990
	mouse hybridoma TSH-5.07	70.5	2.8	Franek, 1991
	GS-transfected NS0 myeloma (amplified)	350	6.5	Brown, 1992
	Fed-Batch Culture Feeds			
glucose + glutamine	mouse hybridoma	290	1.9	Reuveny, 1986a
glutamine	mouse hybridoma 9.2.27	250	5	Flickinger, 1990
amino acids	GS-transfected NS0 myeloma (unamplified)	140	1.8	Robinson, 1994a
	GS-transfected NS0 myeloma (amplified)	900	4	Robinson, 1994a
amino acids + vitamins + serum	hybridoma ATCC HB32	140	4	Luan, 1987b
serum-free multinutrient feeds (serum-free basal medium)	mouse hybridoma CRL 1606 (serum containing basal medium)	550	11	Xie, 1994a,b
	mouse hybridoma	200	4	Noe, 1993
	heterotrioma (human MAb)	750	7.5	Maiorella, 1992a
	hybridoma (murine MAb)	1000		Maiorella, 1992a
	GS-transfected NS0 myeloma (amplified)	895	2.6	Hassell, 1992
	GS-transfected NS0 myeloma (amplified)	560	1.6	Hassell, 1992
	GS-transfected NS0 myeloma (unamplified)	865	8.5	Robinson, 1994a
	GS-transfected NS0 myeloma (amplified)	1800	12.5	Robinson, 1994a
	GS-transfected CHO (unamplified)	110		Hassell, 1992
	GS-transfected CHO (amplified)	250		Hassell, 1992
complete concentrated (serum-free) medium	GS-transfected NS0 myeloma (unamplified)	365	3.65	Bibila, 1994a
	GS-transfected NS0 myeloma (amplified)	1000	7	Bibila, 1994a

cell enumeration assays give reasonable correlations with cell viability, although the correlation varies with the cell type tested (Martin and Clynes, 1993).

Overall, the common host cell lines, such as CHO, SP2/0, and NS0, and most, if not all, hybridoma cell lines can be propagated in serum-free or even protein-free medium, given some adaptation period. The genes coding for the heavy and light antibody chains can be transfected into host cell lines and high expressor clones selected. The glutamine synthetase (GS) vectors (CellTech Ltd.) have been used by many researchers to develop highly expressing cell lines. These cell lines can grow in glutamine-free medium, as well as scavenge ammonia, which greatly simplifies medium and fed-batch process design. Efficient fed-batch process development requires the analysis of spent cell culture medium for limiting and residual nutrients.

Nutrient Fortification in Batch Culture

Early efforts to extend culture longevity focused on supplementing the basal medium with single limiting components, i.e., fortifying the basal medium. These simple modifications of basal media, such as high-glucose DMEM, resulted in relatively modest increases in culture longevity. More detailed medium analysis has led to the development of highly fortified basal media enriched in multiple nutrient components. In the best cases, these fortified media achieve 2–8-fold increases in MAb yields in batch culture relative to unfortified media, with final antibody titers of more than 400 mg/L (Luan et al., 1987b; Jo et al., 1990, 1993a; Franek and Dolnikova, 1991). Some examplee are given in Table 1.

However, many medium components can inhibit cell growth when they are added at levels significantly greater than that commonly found in basal medium. For example, lipoproteins added at levele 4–5-fold over basal concentratione aleo suppressed NS0 cell growth in batch culture (Robinson et al., 1994a). Excessive levels of nutrients can also lead to increased production of inhibitory byproducts, as will be discussed later.

Therefore, although basal medium fortification offers the advantage of the batch mode of operation, the inhibitory effects of high concentrations of some medium components may limit the extent of optimization that can be performed with this strategy. In this case, nutrients have to be fed to the culture gradually, i.e., in a fed-batch mode.

Development of Nutrient Feed Solutions

Initially simple fed-batch strategies, e.g., supplementing the culture with glucose or glutamine as the cells near the stationary phase, have evolved into more complex strategies employing multicomponent feeds at various stages of cell growth. As summarized in Table 1, several investigators have reported on the beneficial effects of feeding glucose, glutamine, and/or concentrated amino acid solutions to MAb-producing cultures, achieving final titers of up to 600 mg/L, a 2–4-fold improvement over batch culture. Today, MAb titers between 0.5 and 2 g/L have been achieved in serum-free fed-batch cultures using various approaches for the design of multicomponent feed solutions. Although the strategies employed vary, each relies upon a combination of physiological reasoning, nutrient depletion analysis, and iterative feed design to maximize cell growth, culture longevity, and MAb production. An exception to the iterative nature of fed-batch process design is the use of complete medium concentrates, where fed-batch processes can be developed with minimal medium analysis.

A first step in fed-batch process design is to develop or identify a near optimal basal medium (Robinson et al., 1994a). Nutrients are then maintained at a constant concentration during the course of the culture by the addition of concentrated nutrient solutione designed to match the nutritional requirements of the cells as determined by the analysis of spent culture medium. Reiterative depletion analysis of spent culture medium allows for the fine tuning of specific supplements for the growth and maintenance phasee of the culture. For example, a preliminary serum-free fed-batch culture of a GS-transfected unamplified NS0 cell line, where only amino acids and glucose were replenished, yielded 140 mg of MAb/L, an approximately 75% improvement over batch culture (Robinson et al., 1994a). Multiple nutrient components, as well as complete concentrated basal medium, were added back in a series of fed-batch cultures, revealing that the proteinaceous medium components were now limiting. A refined fed-batch culture,

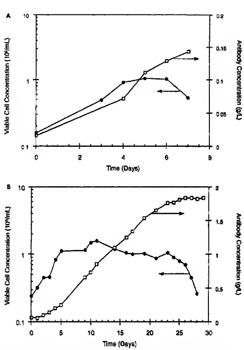

Figure 1. Batch and fed-batch cultures of an amplified GS-transfected NS0 recombinant cell line. Viable cell concentration (●) and monoclonal antibody concentration (□) as a function of elapsed culture time: (A) batch culture; (B) fed-batch culture.

where amino acids, glucose, and proteins were fed, reached a final MAb titer of 0.5 g/L. Finally, multiwell plate growth assays showed that lipoproteins were inhibitory at the levels used in the refined fed-batch culture. Protein-free lipid emulsions were prepared (Seamans et al., 1994) and added together with other medium components. This fed-batch culture yielded 850 mg/L MAb. When a similar process design protocol was applied to an amplified GS-transfected cell line, a final MAb concentration of 1.8 g/L was reached (Figure 1B). Ammonia buildup was limited through the use of glutamine-free medium and the action of the selectable marker, GS, which serves to fix ammonia. As shown in Figure 1, only a moderate increase in maximum cell density was achieved in these fed-batch cultures as compared to batch cultures: 1.5×10^6 versus 1×10^6 cells/mL, respectively. However, substantial increases in the culture viability index, as well as the specific MAb secretion rate, resulted in increased final titers (Robinson et al., 1994a). Similar fed-batch approaches have been used by other investigators to reach MAb titers of 0.5 to greater than 1.5 g/L in serum-free cultures of various transfected NS0 and CHO cell lines (Hassell et al., 1992; Brown et al., 1992; Reff, 1994) or various hybridoma cell lines (Noe et al., 1994).

Xie and Wang (1994a,b) used a model of cellular stoichiometry based on the estimated cell composition (protein, DNA, RNA, lipids, carbohydrates), product composition (amino acids), vitamin yields, and ATP demand to design nutrient feeds, while glucose and glutamine were maintained at low concentrations. The culture feed rate was determined by the measured cell density and estimated growth rate. With this approach, they achieved increases in the viable cell density and final MAb titer of up to 2- and 10-fold over batch culture, respectively, reaching a final concentration of 0.5 g of MAb/L in tissue culture flasks and 0.9 g/L in controlled bioreactors.

Identification of nutrients that stimulate productivity when acting in concert is very important for fed-batch feed formulation. Statistical experimental design, combined with an analysis of the underlying metabolic pathways, can identify such synergistic components (Glacken et al., 1988). In addition, cultures, can be fed on the basis of on-line measurements of culture performance, such as the oxygen uptake rate (Zhou and Hu, 1994). The effect of environmental parameters, e.g., pH and osmolarity, on cell growth and MAb production can be exploited in multilevel control strategies. On the basis of these principles, Maiorella (1992a) developed the most sophisticated feeding protocols to date. Combinations of glucose, amino acids, phospholipid precursors (choline, ethanolamine), disulfide exchange reagents, vitamins, and trace elements were added to serum-free cultures of human and murine hybridomas to maintain maximum viable cell densities of approximately 1.5×10^6 cells/mL for 300 h and reach final antibody titers of 0.75 and 1 g/L, respectively, in serum-free culture (Howarth et al., 1991; Maiorella, 1992a).

In contrast to the previous strategies, another approach, the use of nutrient feeds in the form of concentrated complete medium, eliminates the labor and time associated with the identification of limiting nutrients and formulation/optimization of nutrient feeds. This technique can be used to quickly improve antibody titers in the early stages of fed-batch culture development. Addition of a concentrated feed solution, containing all medium components except salts and glucose at 10-fold their basal levels, resulted in up to 3.5-fold increases in the culture viability index, 3-fold increases in the specific MAb secretion rate, and 7-fold increases in the final MAb titers, respectively, compared to batch culture (Bibila et al., 1994a). Supplementation with complete concentrated medium also increased culture longevity and heterologous protein titers for other cell lines, such as CHO and 293 cells (Hettwer et al., 1991; Hu et al., 1992). Jo et al. used repeated feeding of a 50-fold concentrated RPMI 1640-based basal medium, together with 10% FBS, glucose, and glutamine, to maintain cells in semicontinuous (constant volume) fed-batch culture for over 100 days. The MAb concentration was maintained over 1 g/L for a period of more than 1000 h (Jo et al., 1993b). Despite its effectiveness, the use of concentrated medium has the disadvantage of high cost, as well as the risk of increasing medium osmolarity and the concentrations of medium components and/or growth byproducts to toxic or inhibitory levels (Bibila et al., 1994a). Nevertheless, as discussed earlier, complete medium concentrates can be used to identify additional limiting components, whose residual concentrations cannot otherwise easily be determined (Bibila et al., 1994a; Robinson et al., 1994a).

Although nutrient feeding is the primary means of prolonging culture longevity, other culture parameters can have an effect as well. Hybridoma culture longevity was increased at low dissolved oxygen concentrations and low temperatures, less than 37 °C (Macmillan et al., 1987; Sureshkumar and Mutharasan, 1991). These effects should also be considered when optimizing fed-batch culture conditions.

In summary, supplementation with complete medium concentrates can quickly improve MAb yields without detailed nutrient analysis. In those cases where glucose or glutamine is limiting, addition of these single compo-

nents leads to increased final MAb concentrations. However, the highest MAb titers have been achieved through a reiterative process of nutrient depletion analysis and the formulation of multicomponent feed solutions.

Optimization of the Antibody Secretion Rate

Most fed-batch culture strategies focus on maximizing the viable cell density and prolonging the stationary phase. Rarely has the specific MAb secretion rate been the focus of optimization. However, culture conditions can strongly influence the specific MAb secretion rate.

Feeding of concentrated nutrient solutions leads to substantial medium osmolarity increases, up to 400 mOsm in the case of long term NS0 fed-batch cultures (Robinson et al., 1994a). The use of complete concentrated medium for feeding results in even higher osmolarity increases (Bibila et al., 1994a). Accumulation of growth byproducts, such as lactate, also contributes to increases in osmolarity (Ozturk et al., 1991a). Although increased osmolarity suppresses cell growth, it can increase the antibody secretion rate. The specific MAb secretion rate increases up to 2.5-fold for cells grown above 300 mOsm and up to 435 mOsm (Ozturk et al., 1991a; Oh et al., 1993; Bibila et al., 1994a). The specific productivity rose nearly 2-fold compared to batch culture as the osmolarity increased in the later stages of NS0 cell fed-batch culture (Robinson et al., 1994a). Solute stress, in general, can lead to increased MAb yields (Maiorella et al., 1989). In culture systems where cell growth and production are separated, the effects of osmotic or other environmental stresses on the specific secretion rate can be utilized to maximize final MAb titers. Alternatively, hybridoma cells can be adapted to high osmolarities to maintain both cell growth and high secretion rates (Oh et al., 1993).

The nutritional environment of the cells and their growth stage also affect MAb secretion. Higher specific MAb secretion rates are, in many cases, observed when hybridoma cells are grown in serum-free and lipid-lean media (Glassy et al., 1988). Auberson et al. (1991) reported that amino acid supplementation alone did not affect the specific MAb secretion rate in hybridoma culture. However, Omasa et al. (1992) observed increases in the secretion rate when hybridoma cells were fed with glutamine, but not when they were fed with other amino acids. The nutrient feed composition and feeding rate can also significantly affect the specific MAb secretion rate in fed-batch hybridoma cultures (Kwang et al., 1991; Xie and Wang, 1994b).

The specific productivity often increases during the stationary and death phases of batch or fed-batch cultures. A series of literature reports suggests that this increase is indeed due to active MAb synthesis by viable cells rather than to cell lysis or shedding of surface-associated MAb (Reddy and Miller, 1992; Robinson et al., 1994b). Although the results appear to be cell line dependent, the specific secretion rate also increases when cells are exposed to DNA synthesis or selected (non-MAb) protein translation initiation inhibitors (Suzuki and Ollis, 1990), a decreasing dilution rate in chemostat (Dean et al., 1987; Miller et al., 1988a; Ramirez and Mutharasan, 1990) and perfusion cultures (Reuveny et al., 1986a; Seaver, 1987), and restricted growth in immobilized systems (Lee and Palsson, 1990). Several kinetic models of hybridoma growth and MAb secretion predict an increase in secretion rates at lower growth rates (Suzuki and Ollis, 1989; Bibila and Flickinger, 1992; Savinell and Palsson, 1992). Based on these observations, the strategy for many fed-batch culture protocols focuses on maintaining cell viability and establishing an extended pseudo steady state stationary phase once the cells reach their maximum cell density (Maiorella, 1992a; Robinson et al., 1994b). In such cases, high final MAb titers can be achieved even in the absence of high maximum cell densities.

The addition of protein inducers or chemical enhancers can also increase the specific MAb secretion rate. For example, an immunoglobulin production stimulating activity was found in chicken egg yolk lipoprotein and a milk product (Murakami et al., 1991). Some cellular proteins, such as immunoglobulin production stimulating factors I and II, also enhance the productivity of MAbs, mainly IgM, by some human hybridoma cell lines by more than 20-fold (Yamada et al., 1989). The specific MAb secretion rate increases up to 2-fold in cultures supplemented with nutrient cocktails, such as OptiMAb (Gibco), which contain secretion enhancers (Broad et al., 1989). However, these supplements might be not only cost prohibitive but also undesirable, particularly given the current trend toward well-defined serum-free and low-protein media. Sodium butyrate, as well, stimulates MAb secretion, presumably by rendering the DNA more accessible to RNA polymerase (Oh et al., 1993). Since sodium butyrate and other enhancers also suppress cell growth, the timing of enhancer addition is critical for optimal MAb production (Chang et al., 1994).

As summarized in Table 2, other environmental parameters, such as pH, temperature, and dissolved oxygen, can affect MAb secretion. Although growth is suppressed at high temperatures (>37 °C), MAb secretion sometimes increases (Sureshkumar and Mutharasan, 1991). Low pH, 6.5–7.0, can also stimulate MAb secretion (Miller et al., 1988b; Ozturk and Palsson, 1991b; Schurch et al., 1992). Moderate levels of dissolved oxygen, 5–25%, can enhance MAb production, particularly in serum-free media (see Table 2).

In some cases, feed conditions that optimize culture longevity have a negative effect on the specific MAb secretion rate. In one example, fortification of the basal growth medium led to substantial increases in the culture viability index (1.8-fold) but no increases in the final MAb titer, due to parallel decreases in the specific MAb secretion rate (Franek and Dolnikova, 1991). A maximum in the viability index–final MAb titer relationship has also been reported for recombinant NS0 myeloma cells grown under different fed-batch culture conditions (Bibila et al., 1994a). Identification of such interrelationships is important for optimal fed-batch culture design.

In general, high osmolarity and low pH decrease cell growth but increase MAb secretion rates. Various nutritional and chemical additives can also increase MAb secretion, most likely in a cell line dependent manner. Temperature, dissolved oxygen, and, perhaps, dissolved carbon dioxide concentrations (Aunins and Henzler, 1993) can also influence specific productivity. Multilevel environmental parameter control of osmolarity, pH, and dissolved oxygen can be used to separately optimize cell growth and MAb secretion in batch and fed-batch hybridoma cultures (Maiorella et al., 1992a; Sheu et al., 1992).

Minimization of Byproduct Accumulation

Besides nutrient limitation, accumulation of metabolic byproducts, in particular lactate and ammonia, in the cell culture medium can also inhibit cell growth and antibody production in fed-batch cultures. Excessive lactate buildup can result in increased medium osmolarity or, in the absence of pH control, decreased culture pH. Ammonia can permeate the cell wall and partition into cellular

Table 2. Effect of Environmental Parameters on Monoclonal Antibody Production in Batch Culture

parameter	cell line	improved titer (mg/L) (parameter value)	control titer (mg/L) (parameter value)	reference
dissolved oxygen	murine hybridoma	200 (25%)	125 (60%)	Reuveny, 1986b
	murine hybridoma	25 (5%)	14 (95%)	Meilhoc, 1990
	murine hybridoma	100 (5 ppm)	5 (0.5 ppm)	Ogawa, 1992
	human hybridoma	30 (5 ppm)	10 (0.5 ppm)	Ogawa, 1992
	hybridoma	53–68 (gradual decrease from 20% to 5%)	20–25 (30%)	Sheu, 1992
	hybridoma	98–109 (gradual decrease from 40% to 5%, fed-batch)	20–25 (30%)	Sheu, 1992
osmolarity	GS-transfected NS0 myeloma (unamplified)	120 (300 mOsm)	100 (270 mOsm)	Bibila, 1994a
	mouse hybridoma 2HG11 (adapted)	245 (350 mOsm)	155 (300 mOsm)	Oh, 1993
	heterotrioma (human MAb)	65 (400 mOsm)	30 (300 mOsm)	Maiorella, 1992a
sodium butyrate	mouse hybridoma 2HG11 (adapted)	170 (0.1 mM)	155 (0 mM)	Oh, 1993
osmolarity + sodium butyrate	mouse hybridoma 2HG11 (adapted)	350 (350 mOsm + 0.1 mM)	155 (300 mOsm + 0 mM)	Oh, 1993

compartments, disrupting the local pH (MacQueen and Bailey, 1990). Table 3 summarizes some of the published growth-inhibitory lactate and ammonia concentrations for a variety of antibody-producing cell lines.

Several strategies can be followed to minimize byproduct accumulation in fed-batch cultures. Controlled addition of glucose and glutamine can minimize lactate and ammonia accumulation, significantly improving culture performance (Glacken et al., 1986; Glacken, 1987). Operating under glutamine-limited fed-batch culture conditions not only reduces ammonia accumulation but also reduces the overflow metabolism of other amino acids and results in increases in the glucose and glutamine yield coefficients (Ljunggren and Häggström, 1992). Substitutions of glucose with other carbon sources such as galactose (Glacken et al., 1989), fructose (Duval et al., 1992), or mannose (Jayme, 1991), and glutamine with alternative amino acids, such as glutamic acid, or slowly hydrolyzed dipeptides (Holmlund et al., 1991) also reduce lactate and ammonia accumulation. However, total glucose substitution by other hexoses can alter antibody glycosylation (Moellering et al., 1990). The GS selection/amplification system and subsequent cell growth in glutamine-free medium can largely eliminate ammonia buildup (Bebbington et al., 1992). For example, ammonia levels remained under 4 mM during long term (2–3 weeks) fed-batch cultures of GS-transfected NS0 cells (Robinson et al., 1994a), well below the reported inhibitory ammonia levels (Bibila et al., 1994b). In addition to lactate and ammonia, the accumulation of other metabolic byproducts, such as alanine (Hettwer et al., 1991), in the culture medium might also inhibit growth. Hybridoma cells have been shown to secrete a significant number of as yet unidentified low and high molecular weight inhibitory components as well (Siwiora et al., 1994). Removal of growth byproducts by ion exchange techniques (Carbonne et al., 1992; Thommes et al., 1991) or electrodialysis (Chang et al., 1994), use of ammonia detoxifiers, such as potassium ions (Martinelle and Häggström, 1994), adaptation of cells to high levels of lactate and ammonia (Inlow et al., 1992; Schumpp and Schlaeger, 1992), and expression of desirable metabolic activity, such as that of glutamine synthetase (Brown et al., 1992), offer potential alternatives for minimizing the effects of byproduct accumulation.

In conclusion, minimization of inhibitory growth byproducts is essential to reach high cell densities in fed-batch systems. Although several strategies can minimize lactate and ammonia accumulation, only a few, such as slow substrate feeding, can be implemented reliably on a large scale. The GS expression system reduces ammonia accumulation and is gaining increasing popularity. The potential of techniques such as substrate substitution, cell adaptation, or metabolic pathway engineering will most likely have to be evaluated on an individual cell line basis. In the future, identification and characterization of inhibitory byproducts other than lactate and ammonia and the development of methods to reduce their inhibitory influence may lead to further improvements in fed-batch culture performance.

Product Quality Issues

Product quality is, in many aspects, as important as the product titer achieved in fed-batch cultures. Culture conditions can significantly affect product quality. In general, the proteolysis of MAbs in culture medium is not a significant problem, since the proteinases that have been identified in hybridoma culture conditioned media are inactive at neutral pH (Karl et al., 1990). However, Mohan et al. recently observed the degradation of an MAb during batch culture of murine hybridoma cells (TB/C3), suggesting that some cell lines may produce a protease active at neutral pH (Mohan et al., 1993). In addition, the asparagine and glutamine residues of proteins, including IgG (Tsai et al., 1993), can deamidate, even under mild culture conditions. Such deamidation increases the charge heterogeneity and could, potentially, alter *in vitro* activity (Liu, 1992).

Undetermined changes in the environmental conditions that arose when producing human IgM or murine IgG in ascites fluid, serum-containing, or serum-free *in vitro* cell cultures were shown to profoundly impact product quality as defined by *in vitro* activity and *in vivo* residence times (Patel et al., 1992; Maiorella et al., 1993a). Human IgM produced in ascites had an increased *in vivo* circulatory half-life, but reduced binding

Table 3. Effects of Lactate and Ammonia on the Growth of Antibody-Producing Cells

cell line	inhibitory lactate levels (mM)[a]	inhibitory ammonia levels (mM)[a]	reference
mouse hybridoma CRL-1606	40	5	Glacken, 1987
mouse hybridoma VII H-8	>22	3	Reuveny, 1987
mouse hybridoma AB2-143.2	no inhibition up to 40 mM		Miller, 1988b
mouse hybridoma SB-4082		1.8	Truskey, 1990
mouse hybridoma ATCC TIB 131		≈10	MacQueen, 1990
mouse hybridoma 167.4G5.3	55	4	Ozturk, 1992
GS-transfected NS0 myeloma			
unamplified	>30 mM		Bibila, 1994b
amplified	>30 mM		Bibila, 1994b

[a] Concentrations reported reduce the cell growth rate to 50% of its maximum value.

activity compared to the same IgM produced in serum-free medium in an air-lift reactor. The cell culture derived IgM had greater *in vitro* stability and a higher degree of sialylation. However, other IgMs produced in serum-free cell culture had longer *in vivo* residence times than the ascites-derived material (Maiorella et al., 1993a). Similarly, murine IgG produced by serum-free culture of hybridoma cells contained a larger percentage of sialylated oligosaccharides than it did when produced in ascites fluid (Patel et al., 1992). Culture conditions such as glucose starvation (Stark and Heath, 1979; Elbein, 1987; Rearick et al., 1981), ammonia inhibition (Maiorella, 1992b), extremes of culture pH (Borys et al., 1993), and cell growth state (Hahn and Goochee, 1992) can all influence the glycosylation of proteins. Finally, the host cell line can also affect protein glycosylation. When the same anti-CD18 MAb was produced in a CHO and an NS0 cell line, the resultant glycosylation profiles were markedly different. In comparison to the CHO-derived MAb, the NS0-expressed MAb carried a much smaller percentage of sialylated *N*-glycans (Ip et al., 1992).

Gramer and Goochee (1993, 1994) also detected glycosidase activities, such as sialidase, β-galactosidase, and fucosidase, in the cell lysates of CHO, 293, NS0, and hybridoma cell lines. Although the sialidase activity of NS0 cells was unstable at culture pH, the sialidase activities of CHO and hybridoma cells were more stable. Accumulation of such glycosidase activity in prolonged fed-batch cultures could impact the final glycosylation state of the secreted antibody.

Robinson et al. (1994b) conducted a detailed study of the effect of extended culture lifetimes, which are common to many fed-batch processes, on the biochemical characteristics of an MAb produced by recombinant NS0 cells. The molecular weight, charge, and antigen binding kinetics of the MAb were constant throughout the course of the culture. However, the glycosylation was shown to be both heterogeneous and variable, with the percentage of MAb with truncated and high-mannose oligosaccharides increasing from 14% early in the culture to nearly 40% late in the culture. Complete lysis of viable cells suggested that the release of potentially incompletely processed MAb by lysed cells represents a negligible fraction of the total MAb secreted, less than 10%. Pulse labeling experiments showed that protein synthesis by the nonviable cells, as determined using trypan blue, was negligible and that the viable cell fraction secreted an increasing percentage of truncated and high-mannose glycoforms of the MAb late in the fed-batch culture.

However, native human IgG is, as well, variably glycosylated; the distribution of glycoforms furthermore varies as a function of age (Parekh et al., 1985, 1988). With the exception of antibodies containing variable region oligosaccharide modifications (Wallick et al., 1988), glycosylation does not affect antigen binding, although it may play a role in eliciting effector functions (Dorai et al., 1991). The *in vivo* circulatory half-life, and thereby the activity of a protein, may be profoundly influenced by its glycosylation, as has been shown for erythropoietin and tissue plasminogen activator [as reviewed in Goochee et al. (1991)]. However, there are, to date, no reported studies on the influence of IgG glycosylation on its serum half-life. The mean residence times of both a human IgM (Maiorella et al., 1993a) and a human IgG (Drobyski et al., 1991) produced in cell culture were similar to immunoglobulins isolated from human serum, indicating that cell culture conditions, *per se*, do not reduce the circulatory half-lives of MAbs. Even though the relationship between an antibody's biochemical characteristics and its pharmacokinetics has not been fully determined, the production of a consistent product remains an important issue from a regulatory viewpoint. Equally important is the necessity to demonstrate product equivalence upon implementing process changes (Maiorella, 1993b).

Fed-Batch Process Monitoring and Control

Due, in part, to the complexity of animal cell metabolism and the poor understanding of the intracellular factors that regulate product synthesis and secretion, there are few studies on the monitoring and control of mammalian cell fed-batch cultures. As a result, the optimization of parameters such as the timing and mode of addition of nutrient feeds is typically performed empirically. For example, increased feeding frequency resulted in substantial improvements in final MAb titers in recombinant NS0 cultures (Noe et al., 1994). Continuous nutrient feeding of NS0 cultures did not yield further increases in antibody concentration (Bibila et al., 1994a). However, Noe et al. (1994) observed increases in final MAb titers in fed-batch cultures of hybridoma cells by changing from daily to continuous feeds.

Systematic approaches for the optimization of fed-batch mammalian cell culture operations can be classified, as for microbial systems, into two general categories: open-loop and closed-loop approaches. In open-loop systems, the culture is fed on the basis of optimal feeding trajectories, as predetermined by mathematical models (optimal control theory). This dynamic programming approach is therefore dependent on the development of kinetic models that describe cell growth and product formation and is only as good as the models used to describe the system. In general, these models can be classified as either unstructured or structured. There are several examples of unstructured kinetic models for hybridoma growth and MAb production reported in the literature (Glacken, 1987; Glacken et al., 1989; Dalili and Ollis, 1990). Using one such model, Glacken et al. (1989) determined the optimal flow rate of medium feed and achieved a greater than 10-fold increase in the cell and MAb yields on serum compard to standard batch cultures. Using similar unstructured kinetic models, De Tremblay et al. (1992, 1993) and Nielsen et al. (1991,

1992) optimized nutrient feeding trajectories in fed-batch cultures of hybridoma cells.

Structured kinetic models have also been used to develop feeding strategies in fed-batch hybridoma cultures (Batt and Kompala, 1989; Batt, 1991; Barford et al., 1992a,b). Although these models are still unable to capture the full complexity of cellular metabolism and product formation, recent models describing the MAb secretory pathway (Bibila and Flickinger, 1992) and the dependence of MAb secretion on the cell cycle (Suzuki and Ollis, 1989) have contributed to a better understanding of intracellular factors that might be important for the development of optimal feeding strategies and bioreactor design.

The closed-loop, or feedback control, approach eliminates the requirement for a process model. Instead, cultures are fed on the basis of on-line measurements of cell performance. Glacken and co-workers (1986) estimated the cell density on the basis of an indirect calculation of the volumetric ATP production rate from the measured oxygen uptake and lactate production rates. Feed rates were adjusted to maintain glucose and glutamine concentrations at low levels and minimize the amount of lactate and ammonia produced in fed-batch hybridoma cultures (Fleischaker, 1982; Glacken et al., 1986). Similar strategies have been used by other investigators (Maiorella et al., 1992b; Ramirez and Mutharasan, 1990; Hu et al., 1990, 1992). Carbon dioxide measurements were also used to maintain hybridoma cells at a constant specific growth rate in fed-batch culture (De Tremblay et al., 1993).

The application of feedback control is limited by the lack of reliable and sterilizable on-line sensors that can adequately monitor culture performance. On-line sensors for pH, dissolved oxygen, and carbon dioxide have been used for indirect estimation of culture growth and metabolic parameters and the development of multilevel control strategies for the optimization of fed-batch hybridoma culture performance, as discussed earlier (Sheu et al., 1989; Maiorella et al., 1992a). Continuous monitoring of the oxygen uptake rate by off-gas analysis is marginally accurate and problematic, because of the slow gas flow rates typical of mammalian cell reactors. Alternatively, the oxygen uptake rate can be determined either by the dynamic method or by using a known mass transfer coefficient (Glacken et al., 1989; Matanguihan et al., 1993; Zhou and Hu, 1994). As is the case for the oxygen uptake rate, monitoring of the CO_2 production rate by off-gas analysis is difficult, especially in bicarbonate-buffered media.

Although on-line instrumentation for cell culture has advanced in recent years, more work is necessary before on-line biosensors can be used routinely and reliably in industrial settings and large scale production systems. Probes for cell mass measurement by culture medium capacitance or optical density suffer from low sensitivity, nonlinear response, and high noise. Laser turbidity probes provide better sensitivity and near linear response, but are insensitive to cell viability (Konstantinov et al., 1992) and may be affected by aeration. Probes that measure NAD(P)H fluorescence (MacMichael et al., 1987) can monitor the metabolic and energy states of hybridoma cells (Siano and Mutharasan, 1991). These measurements are, however, subject to problems of low sensitivity at high cell densities ($>10^6$ cells/mL), interference by culture medium components such as amino acids and proteins (MacMichael et al., 1987), and variability of response under different aeration and agitation conditions (Coppella and Rao, 1990). Probes for on-line measurements by acoustic resonance densitometry (Kilburn and Griffiths, 1989) and spectrocolorimetry (Geahel et al., 1989), as well as by NIR and NMR spectroscopy, are in the development stage.

On-line flow injection analysis (FIA) has been used in conjunction with novel biosensors for real time monitoring of cell culture nutrients and/or byproducts (Graf and Schuegerl, 1991). Immobilization of the enzymes glucose oxidase, glutaminase or glutamate oxidase, and lactate oxidase has been used to develop glucose, glutamine, and lactate biosensors, respectively, used for FIA (Cattaneo et al., 1992; Meyerhoff et al., 1993). FIA potentially could be used for on-line analysis of amino acids or MAbs (Krips et al., 1991) by HPLC. Issues such as the stability of the immobilized enzymes, possible interference by other medium components, and reliable aseptic sampling will have to be addressed before these detection methods can be widely applied.

Although progress has been made in recent years on the development of systematic approaches for mammalian fed-batch culture control and optimization, the field is still in its infancy compared to microbial fermentation control and optimization. Application of optimal control theory and feedback control is dependent on the development of accurate mathematical models and reliable on-line biosensors, respectively. As our knowledge of mammalian cell metabolism and secretory mechanisms expands, and as the technology for the design of reliable on-line biosensors advances, increasingly sophisticated control algorithms and expert systems will play a major role in fed-batch process optimization.

Comparison to Other Bioreactor Operation Modes

Fed-batch culture can substantially increase the final MAb concentration and culture volumetric productivity, as compared to batch culture, while keeping the relative simplicity and reliability of batch stirred-tank operation. The highest antibody titers reported to date in fortified batch cultures are about 2–4-fold lower than those reached in fed-batch cultures: 0.5 versus 1.8 g of MAb/L, respectively. However, the higher titers of fed-batch cultures must be weighed against the extra labor and equipment and possible contamination associated with nutrient additions.

Antibodies have been produced in bioreactors operating in a variety of other modes, including cyclic batch culture (draw–fill operation), batch culture with periodic medium replenishment (semicontinuous culture) (Reuveny et al., 1986a), repeated batch culture (semicontinuous perfusion), and continuous culture, which includes chemostat and perfusion cultures (Griffiths, 1990; Kitano et al., 1986; Robinson and Memmert, 1991). These animal cell technologies have been scaled up to 15 000 L for batch and fed-batch cultures, 2000 L for chemostat cultures, and 400 L for perfusion processes (Werner et al., 1992).

Perfusion culture has been studied extensively, and hybridoma cells have been grown in many different types of perfusion systems (Griffiths, 1990; Mizrahi, 1989): suspension cultures with spin filters (Himmelfarb et al., 1969), suspension cultures with gravitational settling devices (Kitano et al., 1986; Batt et al., 1990), membrane reactors (Schumpp and Schlagger, 1989), hollow fiber reactors (Hirschel and Gruenberg, 1987), ceramic cartridges (Lydersen, 1987), and immobilized, microencapsulated, or entrapped cell systems (Dean et al., 1987; Nilsson, 1987; Rupp et al., 1987; Tyler, 1990). In a general comparison of the different aspects of batch (including fed-batch) versus continuous (including perfusion) operation modes, Noe et al. (1992), Werner et al.

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Table 4. Comparative Performances of Batch, Fed-Batch, and Perfusion Cultures for Different MAb-Producing Cell Lines

	batch		fed-batch		perfusion		
cell line (system)	titer (mg/L)	productivity (mg/L/day)	titer (mg/L)	productivity (mg/L/day)	titer (mg/L)	productivity (mg/L/day)	reference
hybridoma (stirred tank)	10–50	48				48–72	Tolbert, 1985
hybridoma (stirred tank)	120	15	220	27	400	660	Reuveny, 1986a
hybridoma (stirred tank)	200	13				76	Birch, 1987
hybridoma (immobilized system)				70		600	Dean, 1987
hybridoma (hollow fibers)						240–360	Dean, 1987
hybridoma (stirred tank)		7.5				200	Bartley, 1992
NS0 (stirred tank)	145	21	1800	64	450	190	Robinson, 1994a

(1992), and Griffiths (1992) reached the following conclusions: (1) capital investment aspects do not seriously influence the decision between batch or continuous processes; (2) a continuous process results in an increased number of bulks/harvests, leading to increased analytical costs and potential regulatory and licensing problems; (3) a batch/fed-batch operation is more flexible and is easier to implement in existing facilities; and (4) process development and validation of continuous processes require a substantially longer time period.

The cell densities achieved in perfusion culture (10^7–10^8 cells/mL) are typically 1 or 2 orders of magnitude higher than those reached in fed-batch cultures ((1–5) $\times$ 10^6 cells/mL). Table 4 summarizes the publications where these systems have been directly compared. In general, volumetric productivities are 10-fold higher in perfusion processes as compared to fed-batch cultures. In addition, perfusion systems have increased operational time, minimize product residence time and exposure to potentially adverse culture conditions, such as the action of released glycosidases and proteases, reduce the potential for deamidation, and continuously expose the cells to fresh nutrients while removing growth-inhibitory byproducts. Although MAb titers of up to 1 g/L and 1–5 g/L of capsules have been reported for hollow fiber or ceramic cartridge and microencapsulated systems, respectively (Tyler, 1990), perfusion culture titers are in most cases lower than those obtained in fed-batch culture. Furthermore, rigorous process optimization is required for a successful perfusion operation (Yabannavar et al., 1994). Since perfusion processes typically require increased startup and cycle times, equipment failure, including fouling of the retention device (Macmillan et al., 1987; Flickinger et al., 1990), is more frequently encountered. Finally, due to the long cycle times in perfusion cultures, the possibility of genetic drift and contamination is increased. However, despite the challenges associated with large scale perfusion cultures, the first perfusion system for a cell culture product (factor VIII) has been recently approved by the FDA (Boedeker et al., 1994). As the technology for reliable large scale perfusion processes develops, manufacturers will have to choose between the simplicity and high titers of fed-batch processes and the high productivity of perfusion processes accompanied by inherently more difficult facility operation and design.

Conclusions

Fed-batch culture currently represents the most attractive choice for the large scale production of monoclonal antibodies due to its operational simplicity, reliability, and flexibility for implementation in multipurpose facilities. Extension of cell culture longevity and the maintenance of high specific antibody secretion rates through nutrient supplementation, reduction of waste product formation, and control of environmental conditions are important for the design of high-yield fed-batch processes. Recent developments in medium and fed-batch protocol design, along with advances in genetic engineering techniques for the development of high-producing clones, have resulted in MAb titers on the order of 1–2 g/L in long term (2–3 weeks) serum-free fed-batch cultures. The factors important for controlling product heterogeneity and optimizing product quality still need to be identified in order to address solutions to these potential problems.

Taken together, the literature suggests a general format for the development and optimization of fed-batch cell culture processes. The selection or development of high-productivity cell lines and serum-free basal medium is crucial for further success. Analysis of batch cultures will reveal limiting nutrients and expose potentially inhibitory byproducts. The design of nutrient feed solutions should be guided by a model of cell physiology (stoichiometric and metabolic pathway considerations), relying on some insight of metabolic behavior to minimize byproduct formation. Preliminary fed-batch cultures should be fine-tuned by repeated nutrient analysis and feedback experiments. These early studies can be conducted on a small scale in multiwell plates, shake flasks, and spinner flasks. However, the optimization of environmental parameters, feed rates, and control strategies requires controlled bioreactors.

Given that the current accomplishments in the field have reduced the finished cost of therapeutic MAbs from greater than \$10 000 to less than \$1000 per gram (Maiorella, 1992a), the future for fed-batch process optimization will increasingly focus on the areas of increased volumetric productivity, product quality, monitoring, and control.

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Literature Cited

Auberson, L. C. M.; Clark, D. S.; Blanch, H. W. Primary metabolism and antibody production in hybridomas. In *Extended Abstracts*, American Institute of Chemical Engineers 1991 Annual Meeting, November 17–22, 1991, Los Angeles; American Institute of Chemical Engineers: New York, 1991; Abstract 267a, p 502.

Aulitzky, W.; Schulz, T.; Tilg, H.; Niederwieser, D.; Larcher, K.; Ostberg, L.; Scriba, M.; Martindale, J.; Stern, A.; Grass, P.; Mach, M.; Dierich, M.; Huber, C. Human monoclonal antibodies neutralizing cytomegalovirus (CMV) for prophylaxis of CMV disease: Report of a Phase I trial in bone marrow transplant recepients. *J. Infect. Dis.* **1991**, *163*, 1344–1347.

Aunins, J. G.; Hanzler, H.-J. Aeration in cell culture bioreactors. In *Bioprocessing, v.3 of Biotechnology: A Multi-Volume Comprehensive Treatise*, 2nd ed.; Stephanopoulos, G. N., Vol.

1829956

Ed., Rehm, H.-J., Reed, G., Series Eds. in cooperation with A. Pühler and P. Stadler; VCH: Weinheim, Germany, 1993; pp 223–281.

Barford, J. P.; Phillips, P. J.; Harbour, C. Simulation of animal cell metabolism. *Cytotechnology* **1992a**, *10* (1), 63–74.

Barford, J. P.; Phillips, P. J.; Harbour, C. Enhancement of productivity by yield improvements using simulation techniques. *Anim. Cell Technol.* **1992b**, 397–403.

Bartley, A.; MacLeod, A. J. A comparative study of monoclonal antibody yield using batch, continuous or perfusion suspension culture techniques. In *Animal Cell Technology: Developments, Processes and Products*; Spier, R. E., Griffiths, J. B., Macdonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 376–378.

Batt, B. C. Development of high viable cell density bioreactors for hybridoma cultures. Ph.D. Thesis, University of Colorado, Boulder, CO, 1991.

Batt, B. C.; Kompala, D. S. A structured kinetic modeling framework for the dynamics of hybridoma growth and monoclonal antibody production in continuous suspension cultures. *Biotechnol. Bioeng.* **1989**, *34*, 515–531.

Batt, B. C.; Davis, R. H.; Kompala, D. S. Inclined sedimentation for selective retention of viable hybridoma in continuous suspension bioreactor. *Biotechnol. Bioeng.* **1990**, *6*, 458–964.

Bebbington, C. R.; Renner, G.; Thompson, S.; King, D.; Abrams, D.; Yarranton, G. T. High level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Bio/Technology* **1990**, *10*, 169–175.

Bibila, T.; Flickinger, M. Use of a structured kinetic model of antibody synthesis and secretion for optimization of antibody production systems: I. Steady state analysis. *Biotechnol. Bioeng.* **1992**, *39*, 251–261.

Bibila, T.; Glazomitsky, K.; Ranucci, C. S.; Robinson, D. K.; Silberklang, M.; Buckland, B. C.; Aunins, J. G. Monoclonal antibody process development using medium concentrates. *Biotechnol. Prog.* **1994a**, *10*, 87–96.

Bibila, T.; Ranucci, C. S.; Glazomitsky, K.; Buckland, B. C.; Aunins, J. G. Investigation of NS0 cell metabolic behavior in MAb producing clones. *Ann. N.Y. Acad. Sci., Proceedings of the Biochemical Engineering VIII Engineering Foundation Conference* **1994b**, in press.

Birch, J. R.; Lambert, K.; Thompson, P. W.; Kenney, A. C.; Wood, L. A. Antibody production with airlift fermentors. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 2–20.

Bjare, U. Serum-free cell culture. *Pharmacol. Ther.* **1992**, *53*, 355–374.

Boedeker, B. G. D.; Newcomb, R.; Yuan, P.; Braufman, A.; Kelsey, W. Production of recombinant factor VIII from perfusion cultures: I. Large scale fermentation. In *Animal Cell Technology: Products of Today, Prospects of Tomorrow*; Spier, R. E., Griffiths, J. B., Berthold, W., Eds.; Butterworth-Heinemann: Oxford, UK, 1994; pp 580–583.

Borys, M. C.; Linzer, D. I. H.; Papoutsakis, E. T. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by chinese hamster ovary (CHO) cells. *Bio/Technology* **1993**, *11*, 720–724.

Broad, D. F.; Brown, M. E.; Grant, A. P.; Wood, L. A. Scale-up of mammalian cell culture. In *Advances in Animal Cell Biology and Technology for Bioprocesses*; Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds.; Butterworth-Heinemann: Oxford, UK, 1989; pp 412–416.

Brown, M. E.; Renner, G.; Field, R. P.; Hassell, T. Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system. *Cytotechnology* **1992**, *9*, 231–236.

Carbonell, D.; Besmainou, B.; Capiaumont, J.; Lessart, P.; Nabet, P. Development of strategies for the removal of ammonia from animal cell cultures. In *Animal Cell Technology: Developments, Processes & Products*; Spier, R. W., Griffiths, J. B., Macdonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 166–170.

Cattaneo, M. V.; Luong, J. H. T.; Mercille, S. Monitoring glutamine in mammalian cell cultures using an amperometric biosensor. *Biosens. Bioelectron.* **1992**, *7*, 329–334.

Chang, D. Y. H.; Grodzinsky, A. J.; Wang, D. I. C. Enhancement of hybridoma growth and productivity by nutrient enrichment and cellular waste removal through electrical means. In *Abstracts of Papers*, 207th National Meeting of the American Chemical Society, San Diego, CA, March 13–17, 1994; American Chemical Society: Washington, D.C., 1994, 7-BIOT.

Coppella, S. J.; Rao, G. Practical Considerations in the measurement of culture fluorescence. *Biotechnol. Prog.* **1960**, *6* (5), 398–401.

Dalili, M.; Sayles, J. D.; Ollis, D. F. Glutamine-limited batch hybridoma growth and antibody production: experiment and model. *Biotechnol. Bioeng.* **1990**, *36*, 74–82.

Dean, R. C.; Karkare, S. B.; Phillips, P. G.; Ray, N. G.; Runstandler, P. W. Continuous cell culture with fluidized sponge beads. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 145–167.

De Tremblay, M.; Perrier, M.; Chavarie, C.; Archambault, J. Optimization of fed-batch culture of hybridoma cells using dynamic programming: single and multi feed cases. *Bioproc. Eng.* **1992**, *7*, 229–234.

De Tremblay, M.; Perrier, M.; Chavarie, C.; Archambault, J. Fed-batch culture of hybridoma cells: comparison of optimal control approach and closed loop strategies. *Bioproc. Eng.* **1993**, *9*, 13–21.

Dorai, H.; Mueller, B. M.; Reisfeld, R. A.; Gillies, S. D. A glycosylated chimeric mouse/human IgG1 antibody retains some effector function. *Hybridoma* **1991**, *10*, 211–217.

Drobyski, W. R.; Gottleib, M.; Carrigan, D.; Ostberg, L.; Grebenau, M.; Schran, H.; Magid, P.; Ehrlich, P.; Nadler, P. I.; Ash, R. C. Phase I study of safety and pharmacokinetics of a human anticytomegalovirus monoclonal antibody in allogeneic bone marrow transplant recipients. *Transplantation* **1991**, *51*, 1190–1196.

Duval, D.; Demangel, C.; Miossec, S.; Geahel, I. Role of metabolic wastes in the control of cell proliferation and antibody production by mouse hybridoma cells. *Hybridoma* **1992**, *11* (3), 311–322.

Elbein, A. D. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* **1987**, *56*, 497–534.

Fike, R. M.; Pfohl, J. L.; Epstein, D. A.; Jayme, D. W.; Weiss, S. Hybridoma growth and monoclonal antibody production in protein-free hybridoma medium. *BioPharm.* **1991**, March, 26–29.

Fleischaker, R. J. An experimental study in the use of instrumentation to analyze metabolism and product formation in cell culture. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1982.

Flickinger, M. C.; Goebel, N. K.; Bohn, M. A. Determination of specific monoclonal antibody secretion rate during very slow hybridoma growth. *Bioproc. Eng.* **1990**, *5*, 155–164.

Fouser, L. A.; Swanberg, S. L.; Lin, B.-Y.; Benedict, M.; Kellerher, K.; Cumming, D. A.; Riedel, G. E. High level expression of a chimeric anti-ganglioside GD2 antibody: genomic kappa sequences improve expression in COS and CHO cells. *Bio/Technology* **1992**, *10*, 1121–1127.

Frame, K. K.; Hu, W. S. The loss of antibody productivity in continuous culture of hybridoma cells. *Biotechnol. Bioeng.* **1990**, *35*, 469–476.

Franek, F.; Dolnikova, J. Hybridoma growth and monoclonal antibody production in iron-rich protein-free medium: effect of nutrient concentration. *Cytotechnology* **1991**, *7*, 33–38.

Geahel, I.; Dordet, Y.; Duval, D.; Dufau, A. F.; Hache, J. On line cell density estimation by spectracolorimetry. In *Advances in Animal Cell Biology and Technology for Bioprocesses*; Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds.; Butterworth-Heinemann: Oxford, UK, 1989; pp 134–135.

Genetic Engineering News, Sales of monoclonal antibodies predicted to rise to $3.8 billion by 1998, **1993**, *13*, 3.

Glacken, M. W. Development of mathematical descriptions of mammalian cell culture kinetics for the optimization of fed-batch bioreactors. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1987.

Glacken, M. W.; Fleischaker, R. J.; Sinskey, A. J. Reduction of waste product excretion via nutrient control: possible strate-

gies for maximizing product and cell yields on serum in cultures of mammalian cells. *Biotechnol. Bioeng.* **1986**, *28*, 1376–1389.

Glacken, M. W.; Edema, E.; Sinskey, A. J. Mathematical Description of Hybridoma Culture Kinetics I. Initial Metabolic Rate. *Biotechnol. Bioeng.* **1988**, *32*, 491–506.

Glacken, M. W.; Huang, C.; Sinskey, A. J. Mathematical descriptions of hybridoma culture kinetics III. Simulation of fed-batch bioreactors. *J. Biotechnol.* **1989**, *10* (1), 39–66.

Glassy, M. C.; Tharakan, J. P.; Chau, P. C. Serum-free media in hybridoma culture and monoclonal antibody production. *Biotechnol. Bioeng.* **1988**, *32*, 1015–1028.

Goochee, C. F.; Gramer, M. J.; Andersen, D. C.; Bahr, J. B.; Rasmussen, J. R. The oligosaccharides of glycoproteins: Bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Bio/Technology* **1991**, 9, 1347–1355.

Graf, H.; Schuegerl, K. Some aspects of hybridoma cell cultivation. *Appl. Microbiol. Biotechnol.* **1991**, *35* (2), 165–175.

Gramer, M. J.; Goochee, C. F. Glycosidase activities in chinese hamster ovary cell lysate and cell culture supernatant. *Biotechnol. Prog.* **1993**, *9*, 366–373.

Gramer, M. J.; Goochee, C. F. Glycosidase activities of the 293 cell line, the NS0 cell line, and an antibody producing hybridoma cell line. *Biotechnol. Bioeng.* **1994**, *43*, 423–428.

Griffiths, J. B. Perfusion Systems for Cell Cultivation. In *Large Scale Mammalian Cell Culture Technology*; Lubiniecki, A. S., Ed.; Marcel-Dekker Inc.: New York, 1990; pp 217–250.

Griffiths, J. B. Animal cell culture processes; batch or continuous? *J. Biotechnol.* **1992**, *22*, 21–30.

Hahn, T. J.; Goochee, C. F. Growth-associated glycosylation of transferrin secreted by HepG2 cells. *J. Biol. Chem.* **1992**, *267*, 23982–23987.

Hassell, T.; Brand, H.; Renner, G.; Westlake, A.; Field, R. P. Stability of production of recombinant antibodies from glutamine-synthetase amplified CHO and NSO cell lines. In *Animal Cell Technology: Developments, Processes and Products*; Spier, R. E., Griffiths, J. B., MacDonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 42–47.

Hettwer, D.; Escobar, E.; Fieschko, J. Development of a fed-batch serum-free suspension process for a recombinant CHO cell line. In *Extended Abstracts*, American Institute of Chemical Engineers 1991 Annual Meeting, November 17–22, 1991, Los Angeles; American Institute of Chemical Engineers: New York, 1991; Abstract 267e, p 503.

Himmelfarb, P.; Thayer, P. S.; Martin, H. E. Spin Filter Culture: The propagation of mammalian cells in suspension. *Science* **1969**, *164*, 555–557.

Hirschel, M. D.; Gruenberg, M. L. An automated hollow fiber system for the large scale manufacture of mammalian cell secreted product. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 115–144.

Holmlund, A. C.; Chatzisavido, N.; Bell, S. L.; Lindner-Olsson, E. Growth and metabolism of recombinant CHO cell lines in serum-free medium containing derivatives of glutamine. In *Animal Cell Technology: Developments, Processes & Products*; Spier, R. E., Griffiths, T. B., MacDonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 776–779.

Howarth, W.; Maiorella, B.; Inlow, D. Cell culture medium for enhanced cell growth, culture longevity and product expression. European Patent Number 435,911, 1991.

Hu, W. S.; Oberg, M. G. Monitoring and control of animal cell bioreactors: biochemical engineering considerations. In *Large Scale Mammalian Cell Culture Technology*; Lubiniecki, A. S., Ed.; Marcel Dekker Inc.: New York, 1990; pp 451–481.

Hu, W. S.; Gryte, D.; Kyung, Y. S.; Peshwa, M. V.; Perusich, C.; Vits, H. Toward advanced nutrient feeding in animal cell culture. In *Harnessing Biotechnology for the 21st Century: Proceedings of the Ninth International Biotechnology Symposium*, Crystal City, VA, August 19–21, 1992; Ladisch, M., Bose, A., Eds.; American Chemical Society: Salem, MA, 1992; pp 202–205.

Inlow, D., Maiorella, B.; Snauger, A. E. Methods for adapting cells for increased product production through exposure to ammonia. U.S. Patent Number 5,156,964, 1992.

Ip Yu, C. C.; Miller, W. J.; Oliver, C. N.; Krips, D. M.; Aunins, J. G.; Cuca, G.; DiStefano, D.; Gould, S.; Seamans, T. C.; Silberklang, M.; Mark, G. E.; Ellis, R. E. Glycosylation of a recombinant monoclonal antibody expressed in several cell lines. In *Abstracts of Papers*, 203rd National Meeting of the American Chemical Society, San Francisco, CA, April 5–10, 1992; American Chemical Society: Washington, D.C., 1992.

Jayme, D. Nutrient optimization for high density biological production applications. *Cytotechnology* **1991**, *5*, 15–30.

Jo, E. C.; Park, H. J.; Park, J. M.; Kim, K. H. Balanced nutrient fortification enables high-density hybridoma cell culture in batch culture. *Biotechnol. Bioeng.* **1990**, *36*, 717–722.

Jo, E. C.; Kim, D.; Moon, H. M. Step-fortifications of nutrients in mammalian cell culture. *Biotechnol. Bioeng.* **1993a**, *42*, 1218–1228.

Jo, E. C.; Park, H. J.; Kim, D.; Moon, H. M. Repeated fed-batch culture of hybridoma cells in nutrient-fortified high-density medium. *Biotechnol. Bioeng.* **1993b**, *42*, 1229–1237.

Karl, D. W.; Donovan, M.; Flickinger, M. C. A novel acid proteinase released by hybridoma cells. *Cytotechnology* **1990**, *3*, 157–169.

Kilburn, D. G.; Griffiths, J. B. The use of acoustic densitometry for on-line monitoring of cell mass in mammalian cell bioreactors. In *Advances in Animal Cell Biology and Technology for Bioprocesses*; Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds.; Butterworth-Heinemann: Oxford, UK, 1989; pp 114–121.

Kitano, K.; Shintani, Y.; Ichimori, Y.; Tsukamoto, K.; Sasai, S.; Kido, M. Production of human monoclonal antibodies by heterohybridomas. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 282–286.

Konstantinov, K. B.; Pambayun, R.; Matanguihan, R.; Yoshida, T.; Perusich, C. M.; Hu, W. S. On-line monitoring of hybridoma cell growth using a laser turbidity sensor. *Biotechnol. Bioeng.* **1992**, *40*, 1337–1342.

Krips, D.; Sitrin, R. D.; Oliver, C. N. A very rapid 2 min protein A HPLC assay for monoclonal antibodies. *FASEB J.* **1991**, *5*, A465.

Kwang, W. J.; Kim, J. K.; Choe, T. B. The variation of production rate of monoclonal antibodies in the fed-batch culture of hybridoma. *Kon Kuk J. Gen. Eng.* **1991**, *4*, 21–27.

Lee, G. M.; Palsson, B. O. Immobilization can improve the stability of antibody productivity in serum-free media. *Biotechnol. Bioeng.* **1990**, *36*, 1049–1055.

Liu, D. T. Y. Deamidation: a source of microheterogeneity in pharmaceutical proteins. *Trends Biotechnol.* **1992**, *10*, 364–369.

Ljunggren, J.; Häggström, L. Glutamine-limited fed-batch culture reduces the overflow metabolism of amino acids in myeloma cells. *Cytotechnology* **1992**, *8* (1), 45–56.

Luan, Y. T.; Mutharasan, R.; Magee, W. E. Effect of various glucose/glutamine ratios on hybridoma growth, viability and monoclonal antibody formation. *Biotechnol. Lett.* **1987a**, *9* (8), 535–538.

Luan, Y. T.; Matharasan, R.; Magee, W. E. Strategies to extend longevity of hybridomas in culture and promote yield of monoclonal antibodies. *Biotechnol. Lett.* **1987b**, *9* (10), 691–696.

Lydersen, B. K. Perfusion cell culture system based on ceramic matrices. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 170–192.

MacMichael, G.; Armiger, W. B.; Lee, J. F.; Mutharasan, R. On-line measurement of hybridoma growth by culture fluorescence. *Biotechnol. Tech.* **1987**, *1* (4), 213–218.

Macmillan, J. D.; Velez, D.; Miller, L.; Reuveny, S. Monoclonal antibody production in stirred reactors. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 21–58.

MacQueen, A.; Bailey, J. E. Effect of ammonium ion and extracellular pH on hybridoma cell metabolism and antibody production. *Biotechnol. Bioeng.* **1990**, *35*, 1067–1077.

Maiorella, B. Hybridoma culture—optimization, characterization, cost. In *Harnessing Biotechnology for the 21st Century: Proceedings of the Ninth International Biotechnology Symposium*, Crystal City, VA, August 19–21, 1992; Ladisch, M., Bose, A. Eds.; American Chemical Society: Salem, MA, 1992a; pp 26–29.

Maiorella, B. *In vitro* management of ammonia's effect on glycosylation of cell products through pH control. U.S. Patent 5,096,816, 1992b.

Maiorella, B.; Inlow, D.; Howarth, W. Method of increasing product expression through solute stress. International Patent Publication Number WO89/04867, 1989.

Maiorella, B. L.; Winkelhake, J.; Young, J.; Moyer, B.; Bauer, R.; Hora, M.; Andya, J.; Thomson, J.; Patel, T.; Parekh, R. Effect of culture conditions on the IgM antibody structure pharmacokinetics and activity. *Bio/Technology* **1993a**, *11*, 387–392.

Maiorella, B. L.; Ferria, R.; Thomson, J.; White, C.; Branon, M.; Hora, M.; Henricksson, T.; Trigila, R.; Kunitani, M.; Kresin, L.; Dollinger, G.; Jones, R.; Senyik, G.; Young, J.; Mayer, B.; McEntire, J.; Dougherty, J.; Monica, T.; Goochee, C. Evaluation of product equivalence during process optimization for manufacture of human IgM monclonal antibody. *Biologicals* **1993b**, *21*, 197–205.

Martin, A.; Clynes, M. Comparison of 5 microplate colorimetric assays for in vivo cytotoxicity and cell proliferation assays. *Cytotechnology* **1993**, *11*, 49–58.

Martinelle, K.; Häggström, L. Detoxification of ammonium in bioreactor cultivations. In *Animal Cell Technology: Products of Today, Prospects of Tomorrow*; Spier, R. E., Griffiths, J. B., Berthold, W., Eds.; Butterworth-Heinemann: Oxford, UK, 1994; pp 158–160.

Matanguihan, R. M.; Pambayun, R.; Izuishi, T.; Konstantinov, K. B.; Yoshida, T.; Gryte, D. M.; Hu, W. S. Development of instrumentation and control strategy in bioreactor culture of hybridoma cells. In *Animal Cell Technology: Basic Applied Aspects*; Kaminogawa, S., Ametani, A., Hachimura, S., Eds.; Kluwer: Dordrecht, The Netherlands, 1993; Vol. 5, pp 501–507.

Meilhoc, E.; Wittrup, K. D.; Bailey, J. E. Influence of dissolved oxygen concentration on growth, mitochondrial function and antibody production of hybridoma cells in batch culture. *Bioproc. Eng.* **1990**, *5*, 263–274.

Meyerhoff, M. E.; Trojanowicz, M.; Palsson, B. O. Simultaneous enzymatic/electrochemical determination of glucose and L-glutamine in hybridoma media by flow-injection analysis. *Biotechnol. Bioeng.* **1993**, *41*, 964–969.

Miller, W. M.; Blanch, H. W.; Wilke, C. R. A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH. *Biotechnol. Bioeng.* **1988a**, *32*, 947–965.

Miller, W. M.; Wilke, C. R.; Blanch, H. W. Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture. *Bioproc. Eng.* **1988b**, *3*, 113–122.

Mizrahi, A. Techniques and equipment for animal cell cultivation. In *Advances in Animal Cell Biology and Technology for Bioprocesses*; Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds.; Butterworth-Heinemann: Oxford, UK, 1989; pp 314–321.

Moellering, B. J.; Tedesco, J. L.; Townsend, R. R.; Hardy, M. R.; Scott, R. W.; Prior, C. P. Electrophoretic differences in a MAb expressed in three media. *BioPharm.* **1990**, February, 30–38.

Mohan, S. B.; Chohan, S. R.; Eade, J.; Lyddiatt, A. Molecular integrity of monoclonal antibodies produced by hybridoma cells in batch culture and in continuous-flow culture with integrated product recovery. *Biotechnol. Bioeng.* **1993**, *42*, 974–936.

Moore, G. E.; Hood, D. B. Modified RPMI 1640 culture medium. *In Vitro Cell. Dev. Biol.* **1993**, *29A*, 265–267.

Murakami, H. What should be focused in the study of cell culture technology for production of bioactive proteins. *Cytotechnology* **1990**, *3*, 3–7.

Murakami, H.; Yamada, K.; Shirahata, A. S.; Enomoto, A.; Kaminogawa, S. Physiological enhancement of immunoglobulin production of hybridomas in serum-free media. *Cytotechnology* **1991**, *5*, 83–94.

Nielsen, L. K.; Nilopsrbowo, W.; Reid, S.; Greenfield, P. F.; Modelling growth of and antibody production by hybridomas in glutamine limited suspension cultures. *Prod. Biol. Anim. Cells Cult.* 10th ESACT, **1991**, 626–630.

Nielsen, L. K.; Niloperbowo, W.; Reid, S.; Greenfield, P. F. Avoiding rapid growth at high cell densitiss: a potentially important optimization criterion for hybridoma cultures. *Cytotechnology* **1992**, *9* (1), 21–27.

Nilsson, K. Entrapment of cultured cells in agarose beads. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 95–111.

Noe, W.; Bux, R.; Werner, R.; Berthold, W. Aspects of continuous culture versus batch mode for animal cells in industrial processes. In *Animal Cell Technology: Developments, Processes and Products*; Spier, R. E., Griffiths, J. B., Macdonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 354–359.

Noe, W.; Schorn, P.; Bux, R.; Berthold, W. Fed-batch strategies for mammalian cell cultures. In *Animal Cell Technology: Products of Today, Prospects of Tomorrow*; Spier, R. E., Griffiths, J. B., Berthold, W., Eds.; Butterworth-Heinemann: Oxford, UK, 1994; pp 413–418.

Ogawa, T.; Kamihira, M.; Yoshida, H.; Iijima, S.; Kobayashi, T. Effect of dissolved oxygen concentration on monoclonal antibody production in hybridoma cell cultures. *J. Ferment. Bioeng.* **1992**, *74* (6), 372–378.

Oh, S. K. W.; Vig, P.; Chua, F.; Teo, W. K.; Yap, M. G. S. Substantial overproduction of antibodies by applying osmotic pressure and sodium butyrate. *Biotechnol. Bioeng.* **1993**, *42*, 601–610.

Omasa, T.; Ishimoto, M.; Higashiyama, K.; Shioya, S.; Suka, K. The enhancement of specific antibody secretion rate in glucose and glutamine controlled fed-batch culture. *Cytotechnology* **1992**, *8* (1), 75–84.

Ozturk, S. S.; Palsson, B. O. Effect of medium osmolarity on hybridoma growth, metabolism and antibody production. *Biotechnol. Bioeng.* **1991a**, *37*, 989–993.

Ozturk, S. S.; Palsson, B. O. Growth, metabolic and antibody production kinetics of hybridoma cell culture: effects of serum concentration, dissolved oxygen concentration and medium pH in a batch reactor. *Biotechnol. Prog.* **1991b**, *7*, 481–494.

Ozturk, S. S.; Palsson, B. O. Effects of ammonia and lactate on hybridoma growth, metabolism and antibody production. *Biotechnol. Bioeng.* **1992**, *39*, 418–431.

Page, M. J.; Sydenham, M. A. High level expreseion of the humanized monoclonal antibody Campath-1H in chinese hamster ovary cells. *Bio/Technology* **1991**, *9*, 64–68.

Parekh, R. B.; Dwek, R. A.; Sutton, B. J.; Fernandes, D. L.; Leung, A.; Stanworth, D.; Rademacher, T. W.; Mizuochi, T.; Taniguchi, T.; Matsuta, K.; Takeuchi, F.; Nagano, Y.; Miyamoto, T.; Kobata, A. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature (London)* **1985**, *316*, 452–457.

Parekh, R. B.; Roitt, I.; Isenberg, D.; Dwek, R.; Rademacher, T. Age related galactosylation of the N-linked oligosaccharide of human serum IgG. *J. Exp. Med.* **1988**, *167*, 1731–1736.

Patel, T. P.; Parekh, R. B.; Moellering, B. J.; Prior, C. P. Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody. *Biochem. J.* **1992**, *285*, 839–845.

Ramirez, O. T.; Mutharasan, R. Cell cycle and growth phase-dependent variations in size distribution, antibody productivity and oxygen demand in hybridoma cultures. *Biotechnol. Bioeng.* **1990**, *36*, 839–848.

Ray, N. G.; Karkare, S. B.; Runstadler, P. W. Cultivation of hybridoma cells in continuous cultures: Kinetics of growth and product formation. *Biotechnol. Bioeng.* **1989**, *33*, 724–730.

Rearick, J. I.; Chapman, A.; Kornfeld, S. Glucose starvation alters lipid-linked oligosaccharide biosynthesis in chinese hamster ovary cells. *J. Biol. Chem.* **1981**, *256*, 6255–6261.

Reddy, S.; Bauer, K. D.; Miller, W. M. Determination of antibody content in live versus dead hybridoma cells: Analysis of antibody production in osmotically-stressed cultures. *Biotechnol. Bioeng.* **1992**, *40*, 947–964.

Reff, M. E. High level expression of MAbs in CHO cells. In *Abstracts of Papers*, 207th National Meeting of the American Chemical Society, San Diego, CA, March 13–17, 1994; American Chemical Society: Washington, D.C., 1994; 54-BIOT.

Reid, S.; Randerson, D. H.; Greenfield, P. F. Amino acid determination in mammalian cell culture supernatants. *J. Biotechnol.* **1987**, *1*, 68–72.

Reuveny, S.; Velez, D.; Miller, L.; Macmillan, J. D. Comparison of cell propagation methods for their effect on monoclonal antibody yield in fermentors. *J. Immunol. Methods* **1986a**, *86*, 61–69.

Reuveny, S.; Velez, D.; Miller, L.; Macmillan, J. D. Factors affecting cell growth and monoclonal antibody production in stirred reactors. *J. Immunol. Methods* **1986b**, *86*, 53–59.

Reuveny, S.; Velez, D.; Macmillan, J. D.; Miller, L. Factors affecting monoclonal antibody production in culture. *Dev. Biol. Stand.* **1987**, *66*, 169–175.

Robinson, D. K.; Memmert, K. W. Kinetics of recombinant immunoglobulin production by mammalian cells in continuous culture. *Biotechnol. Bioeng.* **1991**, *38*, 972–976.

Robinson, D. K.; Seamans, T. C.; Gould, S. L.; DiStefano, D. J.; Chan, C. P.; Lee, D. K.; Bibila, T.; Glazomitsky, K.; Munshi, S.; Dougherty, B.; O'Neil-Palladino, L.; Stafford-Hollis, J.; Hollis, G. F.; Silberklang, M. Optimization of a fed-batch process for production of a recombinant antibody. *Ann. N.Y. Acad. Sci., Proceedings of the Biochemical Engineering VIII Engineering Foundation Conference* **1994a**, in press.

Robinson, D.; Chan, C. P.; Yu Ip, C. C.; Tsai, P. K.; Tung, J.; Seamans, T. C.; Lenny, A. B.; Lee, D. K.; Irwin, J.; Silberklang, M. Characterization of a recombinant antibody produced during the course of a high yield fed batch process. *Biotechnol. Bioeng.* **1994b**, *44*, 727–735.

Rupp, R.; Gilbride, K.; Oka, M. Cellular microencapsulation for large side production of monoclonal antibodies. In *Large Scale Cell Culture Technology*; Lydersen, B. K., Ed.; Hanser Publishers: Munich, 1987; pp 81–94.

Savinell, J.; Palsson, B. O. Network analysis of intermediary metabolism using linear optimization. II. Interpretation of hybridoma cell metabolism. *J. Theor. Biol.* **1992**, *154*, 455–473.

Savinell, J. M.; Lee, G. M.; Palsson, B. O. On the orders of epigenic dynamics and monoclonal antibody production. *Bioproc. Eng.* **1989**, *4*, 231–234.

Schneider, Y. J. Optimization of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium. *J. Immunol. Methods* **1989**, *116*, 65–77.

Schneider, Y. J.; Lavoix, A. Monoclonal antibody production in semi–continuous serum- and protein-free culture. *J. Immunol. Methods* **1990**, *129*, 251–268.

Schumpp, B.; Schlagger, E. J. Physiological studies on high cell density culture of different cell lines. In *Advances in Animal Cell Biology and Technology for Bioprocesses*; Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds.; Butterworth-Heinemann: Oxford, UK, 1989; pp 224–229.

Schumpp, B.; Schlaeger, E. J. Growth study of lactate and ammonia double-resistant clones of HL-60 cells. In *Animal Cell Technology: Developments, Processes & Products*; Spier, R. E., Griffiths, J. B., Macdonald, C., Eds.; Butterworth-Heinemann. Oxford, UK, 1992; pp 183–185.

Schurch, U.; Cryz, S. J.; Lang, A. R. Scale-up and optimization of culture conditions of a human heterohybridoma producing serotype-specific antibodies to Pseudomonas aeruginosa. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 446–450.

Seamans, T. C.; Gould, S. L.; DiStefano, D. J.; Silberklang, M.; Mark, G. E.; Robinson, D. K. Use of lipid emulsions as nutritional supplements in mammalian cell culture. *Ann. N.Y. Acad. Sci., Proceedings of the Biochemical Engineering VIII Engineering Foundation Conference* **1994**, in press.

Seaver, S. S. Culture method affects antibody secretion of hybridoma cell. In *Commercial Production of Monoclonal Antibodies*; Seaver, S. S., Ed.; Marcel-Dekker Inc.: New York, 1987; pp 47–71.

Seaver, S. S. Enhancing monoclonal antibodies and hybridoma cell lines. *Cytotechnology* **1992**, *9*, 131–139.

Sheu, R. B.; Reid, S.; Greenfield, P. The biological effects of dissolved oxygen tension on a hybridoma cell line and its control as a means of improving MAb production in a protein-free medium. In *Animal Cell Technology: Developments, Processes & Products*; Spier, R. E., Griffiths, J. B., Macdonald, C., Eds. Butterworth-Heinemann: Oxford, UK, 1992; pp 215–217.

Siano, S. A.; Mutharasan, R. NADH fluorescence and oxygen uptake responses of hybridoma cultures to substrate pulse and step changes. *Biotechnol. Bioeng.* **1991**, *37*, 141–159.

Siwiora, S.; Fingberg, M.; Buntermeyer, H.; Lehmann, J. Inhibitory substances secreted in cell culture media of a recombinant CHO and a hybridoma cell line. In *Animal Cell Technology: Products of Today, Prospects of Tomorrow*; Spier, R. E., Griffiths, J. B., Berthold, W., Eds.; Butterworth-Heinemann: Oxford, UK, 1994; pp 167–169.

Stark, N. J.; Heath, E. C. Glucose-dependent glycosylation of secretory glycoprotein in mouse myeloma cells. *Arch. Biochem. Biophys.* **1979**, *192*, 599–609.

Sureshkumar, G. K.; Mutharasan, R. The influence of temperature on a mouse-mouse hybridoma growth and monoclonal antibody production. *Biotechnol. Bioeng.* **1991**, *37*, 292–295.

Suzuki, E.; Ollis, D. F. Cell cycle model for antibody production kinetics. *Biotechnol. Bioeng.* **1989**, *34*, 1398–1402.

Suzuki, E.; Ollis, D. F. Enhanced antibody production at slowed growth rates: Experimental demonstration and a simple structured model. *Biotechnol. Prog.* **1990**, *6*, 231–236.

Thommes, J.; Biselli, M.; Garske, U.; Wandrey, C. Integrated detoxification: reduction of ammonium concentration by dialysis with cation exchange membranes. In *Animal Cell Technology: Developments, Processes & Products*; Spier, R. E., Griffiths, J. B., Macdonald, C., Eds.; Butterworth-Heinemann: Oxford, UK, 1992; pp 171–175.

Tolbert, W. R.; Lewis, C.; White, P. J.; Feder, J. Perfusion culture for production of mammalian cell biomolecules. In *Large Scale Mammalian Cell Culture*; Feder, J., Tolbert, W. R., Eds.; Academic Press Inc.: New York, 1985; pp 97–123.

Truskey, G. A.; Nicolakis, D. P.; Dimasi, D.; Haberman, A.; Swartz, R. W. Kinetic studies and unstructured models of lymphocyte metabolism in fed-batch culture. *Biotechnol. Bioeng.* **1990**, *36*, 797–807.

Tsai, P. K.; Bruner, M. W.; Irwin, J. I.; Yu Ip, C. C.; Oliver, C. N.; Nelson, R. W.; Volkin, D. B.; Middaugh, C. R. Origin of the isoelectric heterogeneity of monoclonal immunoglobulin in 1B4. *Pharmacol. Res.* **1993**, *10*, 1580–1586.

Tyler, J. E. Microencapsulation of mammalian cells. In *Large-Scale Mammalian Cell Culture Technology*; Lubiniecki, A. S., Ed.; Marcel-Dekker, Inc.: New York, 1990; pp 343–361.

Wallick, S. C.; Kabat, E. A.; Morrison, S. L. Glycosylation of a VH residue of a monoclonal antibody against alpha 1–6 dextran increases its affinity for antigen. *J. Exp. Med.* **1988**, *168*, 1099–1109.

Werner, R. G.; Walz, F.; Noe, W.; Konrad, A. Safety and economic aspects of continuous mammalian cell culture. *J. Biotechnol.* **1992**, *22*, 51–68.

Wood, C. R.; Dorner, A. J.; Morris, G. E.; Alderman, E. M.; Wilson, D.; O'Hara, R. M.; Kaufman, R. J. High level synthesis of immunoglobulins in chinese hamster ovary cells. *J. Immunol.* **1990**, *145*, 3011–3016.

Xie, L.; Wang, D. I. C. Stoichiometric analysis of animal cell growth and its application in medium design. *Biotechnol. Bioeng.* **1994a**, *43*, 1164–1174.

Xie, L.; Wang, D. I. C. Fed-batch cultivation of animals using different design concepts and feeding strategies. *Biotechnol. Bioeng.* **1994b**, *43*, 1175–1189.

Yabannavar, V. M.; Singh, V.; Connelly, N. V. Scale-up of spin filter perfusion bioreactor for mammalian cell retention. *Biotechnol. Bioeng.* **1994**, *43*, 159–164.

Yamada, K.; Akiyoshi, K.; Murakami, H.; Sugahara, T.; Ikeda, I.; Toyoda, K.; Omura, H. Partial purification and characterization of immunoglobulin production stimulating factor derived from Namalwa cells. *In Vitro Cell. Dev. Biol.* **1989**, *25*, 243–247.

Zhou, W.; Hu, W. S. On-line characterization of a hybridoma cell culture process. *Biotechnol. Bioeng.* **1994**, *44*, 170–177.

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